The Proteasome-Associated Deubiquitinase UCHL5/UBH-4 in Proteasome Modulation and as a Prognostic Marker in Gastrointestinal Cancers

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Academic dissertation

To be presented for public examination with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 2, Biomedicum Helsinki 1 (Haartmaninkatu 8, Helsinki), on March 2nd 2018, at 12 noon.

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ISBN 978-951-51-4016-6 (print)
ISBN 978-951-51-4017-3 (PDF)
ISSN 2342-3161 (print)
ISSN 2342-317X (online)
http://ethesis.helsinki.fi

Cover layout by Anita Tienhaara
Hansaprint, Vantaa, 2018

“Many nematodes died to bring us this information.”

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“A Scientist is Always Fine.”

-Joseph Fink and Jeffrey Cranor

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals in the text (I-IV). Original publications have been reproduced at the end of the thesis with the permission of the publishers.

I. Matilainen, O., **Arpalahti, L.**, Rantanen, V., Hautaniemi, S. and Holmberg, CI. Insulin/IGF-1 signaling regulates proteasome activity through the deubiquitinating enzyme UBH-4. *Cell Rep. 3(6):1980-95, 2013.*

II. **Arpalahti, L.***, Saukkonen, K.*, Hagström, J., Mustonen, H., Seppänen, H., Haglund, C. and Holmberg, CI. Nuclear ubiquitin C-terminal hydrolase L5 expression associates with increased patient survival in pancreatic ductal adenocarcinoma. *Tumour Biol. 39(6):1010428317710411, 2017.* *these authors contributed equally to this study

III. **Arpalahti, L.**, Hagström, J., Mustonen, H., Lundin, M., Haglund, C. and Holmberg CI. UCHL5 expression associates with improved survival in lymph-node-positive rectal cancer. *Tumour Biol. 39(7):1010428317716078, 2017.*

IV. **Arpalahti, L.***, Laitinen, A.*, Hagström, J., Mustonen, H., Kokkola, A., Böckelman, C., Haglund, C. and Holmberg, CI. Positive UCHL5 Tumor Expression in Gastric Cancer is Linked to Improved Prognosis. *Resubmitted manuscript.* *these authors contributed equally to this study

Publication I is included in the doctoral thesis of PhD Olli Matilainen (2013, University of Helsinki).

ABBREVIATIONS

AAA^+ ATPase	ATP hydrolase, AAA domain containing
ACF	Aberrant crypt foci
ADRM1	Adhesion regulating molecule 1
Adc17	ATPase dedicated chaperone of 17 kDa
ADP	Adenosine-5'-diphosphate
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALK	Anaplastic lymphoma receptor tyrosine kinase
AMER1	APC membrane recruitment protein 1
APC	Adenomatous polyposis coli gene
ARID1A	AT-rich interaction domain 1A
ATM	ATM serine/threonine kinase
ATP	Adenosine-5'-triphosphate
Blm10	Bleomycin-sensitive 10
BAP1	BRCA1 associated Protein-1
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CA19-9	Carbohydrate antigen 19-9
CA125	Carbohydrate antigen 125
CDK	Cyclin-dependent kinase
CDK8	Cyclin-dependent kinase 8
CDKN2A	Cyclin dependent kinase inhibitor 2A
CEA	Carcinoembryonic antigen
CIMP	CpG island methylation pathway
CIN	Chromosomal instability
CP	Proteasome 20S core particle
CRC	Colorectal cancer
CTNNB1	catenin (cadherin-associated protein), beta 1; β-catenin
DAF	Abnormal dauer formation
DCC	Deleted In Colorectal Carcinoma
Ddi1	DNA damage-inducible 1
DEUBAD	DEUBiquitinase Adaptor
Dsk2	Ubiquitin-domain containing dual-specificity protein kinase 2
Dss1	Deletion of SUV3 suppressor
DNA	Deoxyribonucleic acid

DUB(s)	Deubiquitinating enzyme(s)
E1-E3	Polyubiquitinating enzyme 1-3
Ecm29	Extracellular mutant protein-29
ECM	Extracellular matrix
EGFR	Epithelial growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERK5	Extracellular signal-regulated kinase 5
FAMM	Familial atypical multiple melanoma and mole syndrome
FAP	Familiar adenomatous polyposis
FIGC	Familial intestinal gastric cancer
FOXO	Forkhead box O
GAPPS	Gastric adenocarcinoma and proximal polyposis of the stomach
GC	Gastric cancer
GI	Gastrointestinal
GPC1	Glypican-1
GRP78	Glucose-regulated protein 78
HDGC	hereditary diffuse gastric cancer
HECT	Homologous to E6AP carboxyl-terminus
HER2	Human epidermal growth factor receptor 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HSF2	Heatshockfactor 2
Hsp70	Heatshock protein 70
IDP	Intrinsically disordered proteins
IGF-1	Insulin-like growth factor 1
IIS	Insulin/IGF-1 signaling’
Iκβ-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alfa
IPTG	b-D-1-thiogalactopyranoside
JAB1	Jun Activation Domain-Binding Protein 1
JAMM	JAB1/MPN+/MOV34
KRAS	V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog
lncRNA	Long non-coding RNA
MAP	MYH-associated polyposis
MAPK1	Mitogen-activated protein kinase 1
MHC I	Major histocompatibility complex, class I

MIN	Microsatellite instability
miRNA	MicroRNA
MMR	DNA mismatch repair gene/pathway
MMRs	DNA mismatch repair proteins
MODC	Mouse ornithine decarboxylase
MOV34	Moloney Leukemia Virus-34 Proviral Integration
MPK1	mitogen-activated protein kinase 1
MPN+	Mpr1/Pad1 N-terminal
mRNA	Messenger RNA
MSS	Microsatellite stable
MSI	Microsatellite instability
mTOR	Mechanistic target of Rapamycin
NFRKB	Nuclear Factor Related To KappaB Binding Protein
NF-Y	nuclear factor Y
NLS	Nuclear localization signal
NRAS	Neuroblastoma Ras viral oncogene homolog
Nrf1	Nuclear respiratory factor 1
Nrf2	Nuclear factor (erythroid-derived)-like 2
NQO1	NAD(P)H quinone dehydrogenase 1
ODC	Ornithine decarboxylase
p16	CDKN2A, *i.e.* Cyclin dependent kinase inhibitor 2A
p19	CDKN2D, *i.e.* Cyclin dependent kinase inhibitor 2D
p21	CDKN1A, *i.e.* Cyclin dependent kinase inhibitor 1
p53	TP53, *i.e.* Tumor protein 53
PA200	Proteasome activator 200 kDA
PAC	Proteasome-assembling chaperone
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PIP	Proteasome-interaction protein
PI(3)K	Phosphatidylinositol-3-kinase
PJS	Peutz-Jeghers syndrome
POMP	Proteasome maturation protein
PRU	Pleckstrin-like receptor for ubiquitin
PSME1-4	Proteasome activator subunit 1-4
PTEN	Phosphatase and tension homolog
PTMs	Post-translational modifications
Rad23	Radiation sensitivity abnormal 23

RBR	RING-between-RING
RING	Really interesting new gene
RNA	Ribonucleic acid
RNAi	RNA interference
RP	Proteasome 19S regulatory particle
Rpn	Regulatory particle non-ATPase
Rpt	Regulatory particle triple-ATPase
S5a	26S proteasome non-ATPase regulatory subunit 4
Sem1	Suppressor of Exocyst Mutations 1
siRNA	Small interfering RNA
SKN-1	Skinhead transcription factor 1
SMAD2	Mothers against decapentaplegic homolog 2
SMAD4	Mothers against decapentaplegic homolog 4
SMAD7	Mothers against decapentaplegic homolog 7
SMURF2	SMAD specific E3 Ubiquitin protein ligase 2
SPS	Serrated polyposis syndrome
SNF2	Sucrose NonFermenting 2
TAP1	Transporter associated with antigen processing 1
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment
TORC1	transducer of regulated cAMP response element-binding protein 1
TP53	Tumor protein 53
Ub	Ubiquitin
Ubp6	Ubiquitin carboxyl-terminal hydrolase 6
UBA	Ubiquitin-associated domain
UBA1	Ubiquitin like modifier activating enzyme 1
UBA6	Ubiquitin like modifier activating enzyme 6
UBD	Ubiquitin binding domain
UBH-4	Ubiquitin C-terminal hydrolase 4
UBL	Ubiquitin-like
UBLCP1	Ubiquitin-like containing CTD phosphatase 1
UCH	Ubiquitin C-terminal hydrolase
UCHL1	Ubiquitin carboxyl-terminal hydrolase L1
UCHL3	Ubiquitin carboxyl-terminal hydrolase L1
UCHL5	Ubiquitin carboxyl-terminal hydrolase L5
Uch37	Ubiquitin C-terminal hydrolase 37; UCHL5

UIM	Ubiquitin interacting motif
UPS	Ubiquitin-proteasome system
UPR	Unfolded protein response
Usp	Ubiquitin specific peptidase
USP14	Ubiquitin carboxy-terminal hydrolase-14
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
ZnF-UBP	Zinc finger ubiquitin-specific protease domain

TIIVISTELMÄ

Ubikitiini-proteasomijärjestelmä (UPJ) on tärkeä proteiinien hajotusjärjestelmä, joka yhdessä autofagia-lysosomijärjestelmän kanssa ylläpitää solujen proteiinitasapainoa. UPJ:n katalyyttinen ydin on proteasomi: monimutkainen holoentsyymi, joka koostuu useista alayksiköistä joilla on eri funktioita. UPJ:n toiminnan häiriöt liittivät läheisesti eri tauteihin, kuten moniin ikääntymiseen liittyviin hermorappeumasairauksiin (esim. Alzheimerin, Parkinsonin ja Huntingtonin tauti), ja erilaisisiin syöpiin. UPJ:n merkityksellisyydestä huolimatta sen säätelymekanismit tunnetaan kuitenkin edelleen huonosti, etenkin *in vivo*. Proteasomi-inhibiittoreita (esim. Bortezomib) käytetään yleisesti syöpähoidoissa (esim. multippelimyeloomassa ja vaippasolulymfoomassa), mutta näihin lääkkeisiin liittyvä toksisuus, potentiaalinen resistanssi ja muut haitalliset sivuvaikutukset ovat luoneet akuutin tarpeen vaihtoehtoisille UPJ:tä sääteleville hoitokohteille. Tämän väitöskirjan tarkoituksena oli löytää ja karakterisoida uusia UPJ:n säätelijöitä elävässä, monisoluisessa organismissa, käyttäen mallieläimenä *Caenorhabditis elegans*-sukkulamatoa. Tavoitteena oli lisäksi tutkia projektin aikana tunnistetun UCHL5/UBH-4-proteasomisäätelijän potentiaalista syöpämarkkeriroolia eri ruoansulatuskanavan alueen syövissä.

Projektin ensimmäisessä osassa osoitettiin, että UCHL5/UBH-4, proteasomiin liittyvä deubikitinaasi, eli proteasomisubstraateista ubikitiinia poistava entsyymi, säätelee proteasomin aktiivisuutta *C. elegans*-sukkulamadoilla. *uchl5:n* hiljentäminen (knockdown) lisäsi proteotoksisten proteiinien hajotusta ihmisen syöpäsoluissa. Sukkulamadoilla *ubh-4*-ekspressiota sääteli ikääntymiseen liittyvä insuliini/IGF-1 signalointireitti (IIS) kudos-riippuvaisesti DAF-16/FOXO-transkriptiotekijän kautta. Lisäksi lievä *ubh-4*:n ekspressiotason lasku sai aikaan vähäisen eliniän pidentymisen, vaikuttamatta kuitenkaan sukkulamatojen jälkeläismäärään. Projektin toisessa osassa UCHL5:n tuumori-ekspressiota arvioitiin immunohistokemiallisesti paksusuolensyöpä-, mahasyöpä- ja haiman duktaalikarsinooma-potilaiden kasvainnäytteistä. Korkea UCHL5-immunoekspressio oli yhteydessä kasvaneen eliniän odotteen kanssa potilailla, joilla oli imusolmuke-positiivinen (Dukes C/vaihe III) peräsuolensyöpä. Positiivinen nukleaarinen ja korkea sytoplasminen UCHL5-immunoekspressio liittyivät parempaan ennusteeseen haiman duktaalikarsinoomassa. Mahasyövässä positiivinen UCHL5-immunoekspressio

korreloi nousseen elinajan ennusteen kanssa niillä potilailla, joilla oli pieni kasvainkoko (<5 cm), tai I-II-vaiheen syöpä.

Tässä väitöskirjassa osoitettin, että UCHL5/UBH-4-deubikitinaasi säätelee proteasomin toimintaa ja lisäksi UCHL5 on uusi prognostinen syöpämarkkeri, jolla on potentiaalisesti kliinistä merkitystä. Tulevaisuudessa UPJ:n tunnustettu merkitys eri sairauksien synnyssä ja kehityksessä todennäköisesti vain kasvaa, joten järjestelmän sääntelymekanismien ymmärrys on perustavanlaatuisen tärkeää.

ABSTRACT

The ubiquitin-proteasome system (UPS) is the major cellular pathway for controlled protein degradation, and, together with the autophagy-lysosome pathway, it is a central player in maintaining protein homeostasis. The catalytic core of the UPS is the proteasome, a complex holoenzyme composed of multiple different subunits with varying functions. Disruptions in the UPS are associated with many pathological conditions, including aging-related neurological diseases (such as Alzheimer's, Parkinson's, or Huntington's disease), as well as different cancers. Proteasome inhibitors (*e.g.* Bortezomib) are in use as cancer therapeutics (*e.g.* in refractory multiple myeloma and mantle cell lymphoma), but dose-limiting toxicities, drug-resistance and other adverse side-effects have created an acute need for identifying alternative targets that modulate the UPS. Yet, despite its wide-ranging importance, it remains to be defined how UPS is regulated, especially *in vivo*. The purpose of this thesis was to provide new information on UPS modulation in a living, multicellular organism, with the help of the model organism *Caenorhabditis elegans*. Further, the aim was to investigate the potential role of an identified proteasome regulator UCHL5/UBH-4 as a biomarker in three gastrointestinal cancers: colorectal cancer (CRC), gastric cancer (GC) and pancreatic ductal adenocarcinoma (PDAC).

In the first part of the study, it was established that UCHL5/UBH-4, a proteasome-associated deubiquitinating enzyme (DUB), modulates proteasome activity in *C. elegans*, and additionally increases the degradation of proteotoxic proteins in human cancer cells. In *C. elegans*, UBH-4 expression was demonstrated to be regulated by the ageing-regulating Insulin/IGF-1 signaling (IIS) pathway through the transcription factor DAF-16 in a tissue-specific manner. Further, minor knockdown of *ubh-4* resulted in a short lifespan extension without affecting progeny amounts. In the second part of the study, the role of UCHL5 was investigated in various gastrointestinal cancers. UCHL5 tumorexpression was scored with immunohistochemistry from representative patient tumor samples in CRC, GC and PDAC. UCHL5-immunoexpression correlated with increased survival in the subgroup of patients with lymph node-positive (Dukes C/stage III) rectal cancer. In addition, both positive nuclear and high cytoplasmic UCHL5-immunoexpression associated with better prognosis in PDAC. Positive UCHL5-immunoexpression was also linked to enhanced

survival in the subgroups of gastric cancer patients with small tumors (<5 cm) or stages I-II of the disease.

This thesis identified UCHL5/UBH-4 as a new proteasome modulator, and further UCHL5 as a novel prognostic marker with potential clinical relevance. In the future, the recognized significance of UPS in the development and progress of different diseases is only likely to grow. Therefore, understanding the underlying regulatory mechanisms of UPS function is of fundamental importance.

INTRODUCTION

Protein homeostasis, the dynamic process of protein translation and destruction, is maintained in part through the ubiquitin-proteasome system (UPS), a multifaceted pathway that degrades the majority of all cellular proteins. Dysfunctions in this system are present in several age-related diseases, including many types of cancer. Globally, gastrointestinal cancers remain a serious issue, affecting yearly the lives of millions of people. Despite recent developments in their treatment, there is an acute need for more accurate and reliable prognostic markers in clinical praxis. The purpose of this thesis was to identify and characterize new proteasome regulators in a living, multicellular organism. Further, the aim was to investigate the role of one of the identified regulators, the proteasome-associated deubiquitinating enzyme UCHL5/UBH-4, in three gastrointestinal cancer types: colorectal cancer, gastric cancer and pancreatic ductal adenocarcinoma. The literature review will first discuss the specifics of the ubiquitin-proteasome system, and its various roles in maintaining cellular proteostasis. It will then expand on the three gastrointestinal cancers studied in this thesis, and touch on biomarkers available in these cancer types. The results section will in turn examine the function of UCHL5/UBH-4 in proteasome regulation, and describe the prognostic marker role of human UCHL5 in gastrointestinal cancers.

LITERATURE REVIEW

1. The ubiquitin-proteasome system

Proteins are constructed of long polypeptide chains, forming a diverse group of important biomolecules. They are at the very core of our existence, from complex building blocks to facilitating and carrying out the multitude of processes within our cells that define life as we know it. Cellular protein content is thoroughly regulated, and complications in protein homeostasis (proteostasis) lead to proteotoxicity, different disease conditions, and potentially death. The delicate balance in protein turnover is maintained through the synthetization of new proteins (translation), as well as through the degradation of misfolded or superfluous proteins. The destruction of proteins is performed by two parallel and partially overlapping cellular pathways: the autophagy-lysosome pathway (ALP, or autophagy) and the ubiquitin-proteasome system (UPS). In autophagy, the cytoplasmic components are delivered into lysosomes for degradation, thereby clearing out entire organelles and large protein aggregates, in addition to single proteins [1,2]. In contrast, UPS is the major pathway for e.g. the degradation of irregular or redundant regulatory proteins, thus destroying substrates specifically targeted to the UPS [3]. The two degradation systems connect to and complement each other in complex and yet to be fully defined ways; for example, UPS impairment has been demonstrated to upregulate autophagy in several model systems [4-7].

Briefly, in the UPS, a large constituent of substrates are marked for degradation through their polyubiquitination, which is facilitated by three groups of enzymes: E1-E3 (enzyme 1-3) [8] . These enzymes are divided into their respective classes according to their ubiquitin (Ub) activating, conjugating or ligating capacity. Once a polyubiquitinated protein targeted to the proteasome reaches its destination, it is sequentially deubiquitinated, unfolded and translocated into the proteasome core for destruction. At the heart of the UPS is the proteasome, a multisubunit holoenzyme complex that degrades all UPS substrates [9]. Additionally, another group of proteins known as deubiquitinating enzymes (DUBs) remove polyubiquitin from potential substrates, prior to their degradation [10]. Through its active role in protein turnover, UPS takes part essentially in all cellular processes, including protein quality control, signal transduction, cell-cycle control, DNA repair, transcription and major

histocompatibility complex I (MHC I) antigen presentation, to name but few [11]. Aberrant UPS function is present in a wide array of pathological conditions, including several aging-related and neurodegenerative diseases (*e.g.* Alzheimer's, Parkinson's and Huntington 's disease), viral infections and many types of cancer [12,13].

The proteasome is present in all eukaryotes, archaea and even some eubacteria [11]. At any given time, a large portion of proteasomes are in the nucleus, but they are also found throughout the cytosol and adjacent to membraned organelles, such as mitochondria [14]. Localization of proteasomes is dynamic: populations shift between different cellular compartments, presumably in response to fluctuating proteolytic needs [15]. Active proteasomes have also been discovered outside cells, *e.g.* in the serum or cerebrospinal fluid, and the levels of these extracellular proteasomes are often elevated in cancer, and in response to injury [16-21] .

1.1. Proteasome structure and function

The best characterized form of the proteasome consists of a 20S core particle (CP), joined at one (26S) or both ends (30S) by a 19S regulatory particle (RP or PA700). Eukaryotic proteasomes share generally a high degree of conservation, in both subunit composition and genetic sequence [11]. In mammalian cells, more than 50% of proteasomes appear to exist as free 20S, and only 20%-30% were identified as 26S [22-25]. CPs can form with alternate core subunits, as well as hybrid proteasomes, but in this text the term "proteasome" refers to the canonical 26S proteasome, unless otherwise stated. CP-RP2 (CP with two 19S RPs attached) has a molecular weight of roughly 2.5 MDa, and it is composed of over 50 subunits [26]. Main function(s) of the key subunits are summarized in **Table 1**. Deletion of the 20S core subunits is lethal, resulting in growth arrest and death [27].

Table 1. Key function(s) of the 26S subunits and associated DUBs.

Subunit(s)	Function
Rpn1, Rpn10, Rpn13	Ub receptors
Rpn11	detaching last substrate Ub(s) (DUB)
USP14, UCHL5/UBH-4	detaching substrate Ub (associated DUBs), 19S activation
Rpt 1-6 AAA$^+$-ATPases	binding, unfolding and translocation of the substrate
Rpt2, Rpt3, Rpt5	opening the 20S gate
α-subunits (N-termini)	forming 20S gate structure
β1, β2, β5	catalytic activity (peptide hydrolysis)

Table modified from: Collins and Goldberg, 2017. [28]

1.1.1. 20S core particle

The 20S core particle is the proteolytic unit of the UPS, degrading the multitude of proteasomal substrates that are processed by the system. 20S and 19S structure and the relative locations of their various subunits are depictured in **Figure 1**. The 20S core has a molecular weight of approximately 730 kDa [29]. The canonical CP consists of four coaxially stacked heteroheptameric rings (two outer α-rings, two inner β-rings), forming a barrel-like structure [30]. The seven α-subunits (α1-α7) are all distinct from one another, but share considerable structural similarity [31]. The N-termini of the α-subunits form a 13 Å molecular gate that controls substrate access, as well as provides attachment sites for proteasome activators, including 19S [32]. The N-terminus of the α3-subunit is particularly important for the configuration and opening of the gate, as deletion of α3 results in a permanently open pore [32]. The outer-facing surface of the α-rings form seven binding pockets for the different proteasomal activators, which in turn modulate gate opening [33,34]. Many of the 20S α-subunits contain a nuclear localization signal (NLS), which is important for determining proteasome location in cells [35-37]. The central inner cavity of the 20S contains the actual proteolytic functions of the proteasome [38]. Of the seven β-subunits, only three have catalytic activity: β1, β2 and β5 [38]. The proteolytic specificity of these three subunits are in order: caspase-like (cleavage after acidic residues), trypsin-

like (cleavage after basic residues), and chymotrypsin-like (cleavage after hydrophobic residues) activity [38].

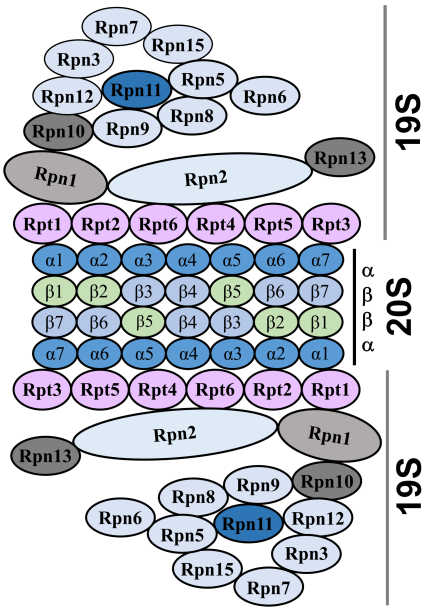

Figure 1. Proteasome structure. Catalytic core subunits marked in green. Image modified from *D'Arcy et al., 2015.* [26]

1.1.2. 19S/PA700 regulatory particle

Because of its closed architecture, the 20S is inherently repressed, and requires activators to facilitate gate opening and substrate degradation; however, essentially any protein that enters the pore can be degraded [11]. The most comprehensively characterized proteasome activator is the canonical 19S RP, which has a molecular weight of approximately 930 kDa [39]. 19S regulates binding, deubiquitination, unfolding and translocation into the core of the various proteasomal substrates [9]. The 19S structure is divided into two subcomplexes: the lid and the base [9]. In the base subcomplex, six AAA^+ ATPase subunits, known as regulatory particle subunits 1-6 (Rpt1-Rpt6), form a heterohexameric ring [40]. They are joined by four regulatory particle non-ATPase subunits (Rpn1, Rpn2, Rpn10/S5a and Rpn13/ADRM1) [40]. The ATPase ring assists in the opening of the 20S gate, and diverts energy from ATP hydrolysis into the unwinding and translocation of proteasome substrates [33,41-45]. The lid subcomplex is a horseshoe-shaped structure, composed of nine different subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15/Dss1/Sem1). In addition, there are several proteins that transiently associate with the RP, which are not considered *de facto* stoichiometric subunits, *e.g.* the proteasome-associated DUBs ubiquitin carboxyl-terminal hydrolase L5 (UCHL5) and ubiquitin carboxy-terminal hydrolase 14 (USP14) (see **Table 1**) [46, 47].

Of the 19S subunits, Rpn1 [48], Rpn10 [49] and Rpn13 [50] recognize and bind ubiquitin. Rpn10 binds ubiquitin through its C-terminal Ub-interacting motifs (UIMs) [51], and Rpn13 with its pleckstrin-like receptor for ubiquitin (PRU)

domain [52,53]. Rpn10 and Rpn13 bind additionally Ub-like (UBL) domains, but their affinity for free ubiquitin is weak [28]. Rpn1 was recently identified as a third Ub-receptor on the proteasome [48], with an affinity for UBL-domains [54,55]. Though both Rpt5 [56] and Rpn15 [57] bind ubiquitin, it is unknown if they function as ubiquitin receptors on the proteasome [9,58]. The roles of the different proteasomal Ub-receptors appear to be both distinct and redundant, and potentially depend on the substrate in question [28]. Whether the Ub-receptors work together, simultaneously, or in sequence, remains to be defined [28].

1.1.3. Alternate proteasome activators

Three different activator families function in 20S gate opening, providing access for proteasomal substrates into the central chamber [11]. The most conserved family of proteasome activators are the eukaryotic 19S, archaeal PAN and eubacterial ARC/Mpa. The other two families are 11S/PA28/REG/PA26 and PA200/Blm1 [11]. These two families share less conservation between species, and their functions and biological relevance is not as well-defined as that of 19S [11]. The common trait for the alternate activators is that they cannot recognize ubiquitin or hydrolyze ATP [59]. Instead, their substrates are small, simple proteins, such as cyclin-dependent kinase (CDK) inhibitors *p16, p19* and *p21* [60]. The 20S can associate at the same time with either one or two 11S or PA200 activators, or in combination with 19S and a second activator, creating hybrid proteasomes [24].

Higher eukaryotes have three different 11S-isoforms: PA28α,β,γ [61]. PA28α (Proteasome activator subunit 1, PSME1) and PA28β (PSME2) assemble into a heteroheptamer ring (PA28αβ, containing three α and four β subunits), and PA28γ (PSME3) is a homoheptamer. The role of 11S in cellular functions is not well understood [11], but the PA28αβ-complex is found for the most part associated with the immunoproteasome [62]. PA28γ has been connected to the degradation of natively unfolded proteins [63-65], and it appears to be of more primordial origin than the two other isoforms, as it is expressed throughout the metazoan line [66]. Hybrid proteasomes with one 11S and 19S are implicated in antigen production for the MHC I complex [24]. PA28αβ is also more abundant in immune tissues and it is upregulated by interferon-γ [67]. Additionally, 11S activators increase the capacity of 20S to degrade oxidized proteins [68].

The 200 kDa monomeric PA200/Blm10 (PSME4) has a dome-like shape [69-71], and it promotes only partial opening of the 20S gate [72]. PA200 is believed to promote degradation of peptides, but not full-sized proteins [11]. The activator is implicated in a variety of processes, including 20S assembly [73], proteasome inhibition [74], DNA repair [69], spermatogenesis [75] and mitochondrial checkpoint regulation [72]. PA200 expression is especially abundant in the testis [76], either forming hybrid proteasomes (19S-20S-PA200), or as single or double-capped complexes (PA200-20S and PA200-20S-PA200) [77]. In addition, PA200 deficient male mice have reduced fertility [77], and PA200 is critically involved in histone degradation during DNA damage and spermatogenesis [77].

1.1.4. Tissue-specific proteasomes

In mammals, constitutive catalytic β-subunits can be replaced upon induction, or tissue-specifically in a variety of immune-related tissues or cells (such as monocytes and lymphocytes) by alternative β1i, β2i, β5i and/or β5t subunits ("i" for immunoproteasome, "t" for thymoproteasome) [78,79]. The βi-subunits of the immunoproteasome demonstrate altered specificity toward cleaving after basic and hydrophobic residues, which is presumed to increase the affinity of the degradation products to the MHC I complex molecules [80]. The immunoproteasome has also been suggested to show a preferential association with the PA28αβ activator [81]. The 26S holoenzyme can contain both constitutive and immune β-subunits simultaneously [79]. Upon viral, bacterial, or fungal infections, up to 90% of constitutive proteasomes in relevant tissues can be replaced by immunoproteasomes [82-84], suggesting that the immunoproteasome can also perform vital housekeeping functions normally governed by constitutive proteasomes [82].

The thymoproteasome has been found only in the cortical epithelial cells of the thymus, shown to play a central role in the maturation of $CD8^+$ T-cells [30]. The catalytic subunits of the thymoproteasome are β1i, β2i and β5t, and the chymotrypsin-like activity of β5t subunit is lower than that of either β5 or β5i [40,79]. The third known tissue-specific proteasome is the spermatoproteasome. In *Drosophila*, two alternate α-subunits (α4T1 and α4T2) are expressed only in the male germline from mid to late stages in spermatogenesis [85-87].

Interestingly, additional 12 proteasome subunits have been found to exist as multiple isoforms in *Drosophila*, with all of the non-canonical isoforms expressed exclusively in the testes (reviewed in [88]). Mammalian testes also have an alternate core subunit, α4s/PSMA8, which is only expressed in spermatids and mature sperm [77,89] in a same complex with immunoproteasome specific β-subunits [77,90]. This demonstrates that by switching a few subunit(s), proteasome function and specificity can be greatly altered, reflecting the dynamic plasticity inherent in the complex.

1.2. Polyubiquitinating enzymes

Ubiquitin, the ubiquitous biomolecule used to tag proteasomal substrates, is a small, 76-aminoacid protein [91,92]. UPS target protein polyubiquitination is a stepwise process facilitated by three classes of enzymes: E1-E3. An additional group of E4 enzymes is also sometimes described; E3s that extend and modify existing ubiquitin chains [93,94]. The polyubiquitination cascade is illustrated in **Figure 2**. To start the process, ubiquitin is activated by an ATP-dependent E1 ubiquitin activator. Of the eight human E1 enzymes, two are associated with the UPS: UBA1 and UBA6 (reviewed in [95]). UBA6 is a fairly recent discovery, described as an ubiquitin activator as late as 2007 [96,97]. Next, one of several dozen human E2s acts as ubiquitin conjugator. In the third group of ubiquitin ligases are approximately 600 different E3s, which confer substrate specificity in the system [98]. Substrate bound E3s recruit Ub-loaded E2s, and facilitate or directly catalyze the transfer of Ub to a target substrate [98]. E3s are divided into three families: homologous to E6AP carboxyl-terminus (HECT) E3 ligases, really interesting new gene (RING) E3 ligases and RING Between RING (RBR) E3 ligases [99]. The RING E3s are the largest family, and they promote approximately 20% of Ub-mediated degradation [100,101]. Together with the RBR E3s, RING E3s use E2 enzymatic activity to transfer the Ub directly to the substrate by acting as a scaffold [98,99]. In contrast, the few dozen HECT E3 ligases catalyze Ub transfer to the substrate independently of E2 enzymatic activity by forming an E3-Ub intermediate as an additional step [98,102-104].

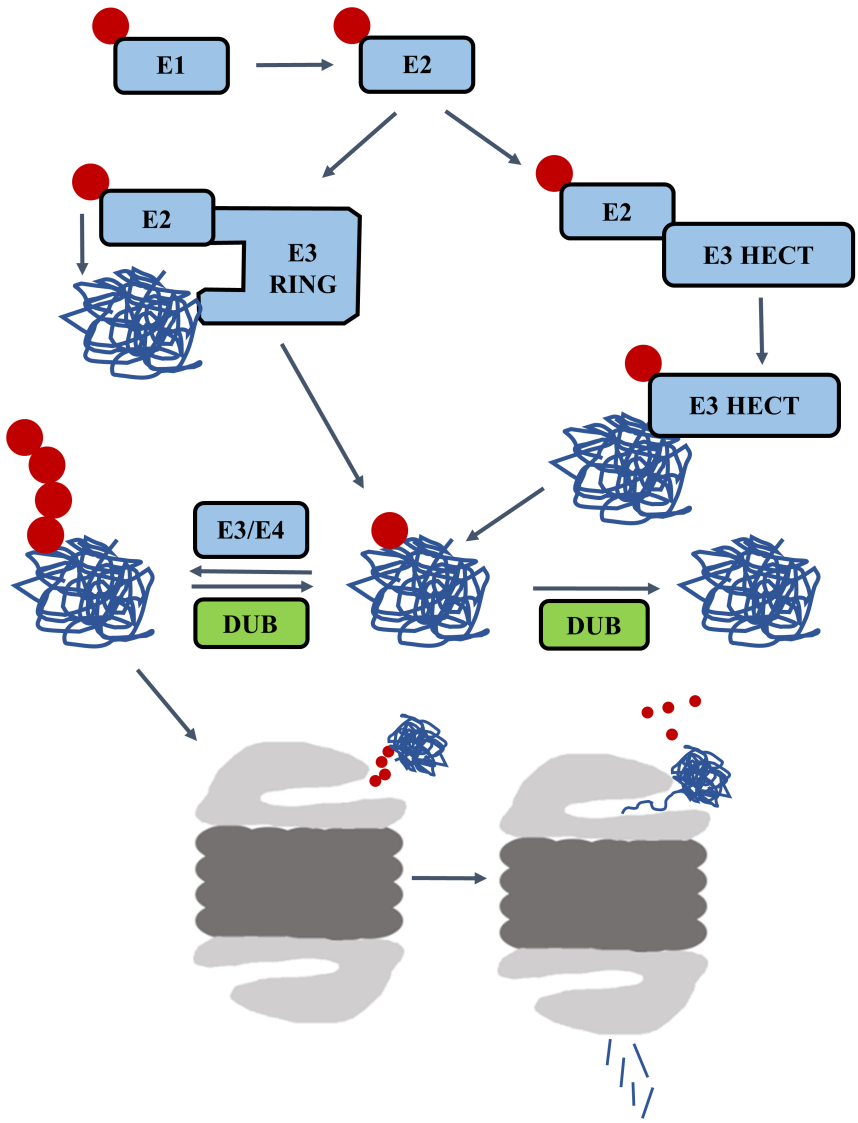

Figure 2. The polyubiquitination process of proteasome substrates. Ubiquitin (Ub, red circles) is activated by E1 (Ub-activating enzyme), requiring ATP hydrolysis. Activated Ub is transferred to an E2 (Ub-conjugating enzyme), and then directly to a substrate (in blue) associated with an E3 RING ligase, acting as a scaffold. Alternatively, Ub can be transferred to an E3 HECT ligase, forming an E3-Ub intermediate, followed by substrate ubiquitination. Deubiquitinating enzymes (DUBs, in green) can remove ubiquitin at multiple steps throughout the process. Components are not in scale. Modified from *Lip et al., 2017.* [99]

Protein ubiquitination status can direct proteins to multiple fates, *e.g.* serving as subcellular localization signals, or degradation tags for both autophagy and UPS [105-109]. Ubiquitination is divided into monoubiquitination, multimono-ubiquitination (attachment of several single ubiquitin moieties into the same molecule), and polyubiquitination [39,108,110-114]. Ubiquitin can form eight different kinds of linkages with itself: either through its seven lysine (K) residues (K6, K11, K27, K29, K33, K48, K63), or its N-terminus [115]. The chains can be homotypic, or contain different linkages (heterotypic), and all assembly forms are potential intracellular targeting signals [115]. Forked chains forming on adjacent lysines of a donor ubiquitin are not recognized by the proteasome [116]. The formation of these undegradable chains can be prevented to some degree by cytosolic ubiquitin interacting motif (UIM) proteins, such as Rpn10 [116]. The canonical signal for proteasomal degradation is a 4-6 moieties long Ub-chain, containing K11 or K48 linkages [92]. K48-linked polyubiquitin chains are also the most abundant ubiquitin tags in cells [117,118]. Additionally, K6-, K27-, K29- and K33-linked polyubiquitin chains can be targeted for proteasomal degradation, although this is not always the case [118]. Ubiquitination on residues other than lysine (cysteine, serine, threonine) [119] or multiple monoubiquitination or diubiquitination may also target substrates to the proteasome [120,121]. Different cellular compartments contain variable pools of E3s and DUBs, which further contributes to make the ubiquitination status of a specific substrate highly context specific [122].

1.3. Deubiquitinating enzymes

Polyubiquitination and even association with the proteasome does not guarantee substrate degradation [123,124]. In a complete or partial reversal of the process mediated by polyubiquitinating enzymes, deubiquitinases remove ubiquitin from target proteins [124]. The human genome codes for approximately 90 Ub-specific DUBs [125-128]. These enzymes cleave the isopeptide bond between the substrate and the primary ubiquitin, detaching the whole chain simultaneously, or an isopeptide bond located between different Ub-molecules in a chain, causing chain trimming [26,122]. Some DUBs display linkage specificity (*e.g.* K48, K63), while others cleave multiple chain types [129]. Several DUBs only become active once they have been recruited to their target location, or bound to a substrate [122]. An integral part of a DUB is its Ub-binding domain (UBD),

consisting of a ubiquitin-interacting motif (UIM), a zinc finger Ub-specific protease domain (ZnF-UBP) [125] and Ub-associated domain (UBA) [130]. Substrate specificity is thought to be conveyed through the Znf-UBP domain [125].

Based on sequence similarity and conservation in their domains, DUBs are divided into six families [131]. These are the JAB1/MPN/MOV34-family (JAMMs), motif-interacting with ubiquitin-containing novel DUB family (MINDYs), Machado–Josephin domain-containing proteases (MJDs), ovarian tumour proteases (OTUs), ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin-specific proteases (USPs) [132]. Five of these families are thiol proteases, or cysteine peptidases, whereas JAMMs are a family of zinc metalloproteases [122,131,133]. UCHs all have a N-terminal catalytic domain, and some of them also contain additional C-terminal domains that facilitate protein-protein interactions [127]. The four human UCHs are UCHL1, UCHL3, UCHL5/Uch37 and BRCA1 associated Protein-1 (BAP1) [122]. The USP family has approximately 60 human members, including the proteasome-associated USP14/Ubp6 [128]. For JAMMs, catalytic activity is conveyed by their JAMM-domain, and members include the proteasome subunit Rpn11 [134,135]. DUBs participate in regulatory cellular processes in many ways. They display variable cellular localization, and can change substrate ubiquitination by directly interacting with E3s [136]. They also act at the proteasome to either hinder or promote degradation of polyubiquitinated substrates [122,136]. One of their fundamental roles is the co-translational processing of pro-ubiquitin: most organisms express ubiquitin as a linear polymer of multiple ubiquitin copies, which needs to be disassembled through DUB activity to generate free monoubiquitin [137,138].

1.4. Proteasome-mediated protein degradation

An estimated 80% of proteins in mammalian cells are degraded by the proteasome, including misfolded or superfluous proteins, in addition to the bulk of recycled housekeeping proteins [139]. Approximately 90% of proteasomal degradation products are 2-10 residues in length [140]. Though the majority of these are further degraded into free amino acids in the cytosol to be reused in the translation of new proteins [13,141,142], some are repurposed in higher

vertebrates by the Transporter associated with antigen processing 1 (TAP1) protein to be loaded onto the MHC I complexes as precursors of antigenic peptides [143]. Proteasome function is flexible, and partial degradation may also result in protein activation (*e.g. p100* in the NF-κβ pathway) [144]. Moreover, not all proteasome substrates arrive to the 20S through the polyubiquitination process [145,146]. Two pathways of protein hydrolysis exist at the proteasome: ubiquitin-dependent and ubiquitin-independent degradation. These two processes are not mutually exclusive, and different populations of the same substrate may be degraded by either process, often depending on the level of substrate unfolding prior to degradation [147].

1.4.1. Ubiquitin-independent protein degradation

Best characterized among proteasome substrates that are degraded ubiquitin-independently is ornithine decarboxylase (ODC), one of the crucial enzymes involved in polyamine biosynthesis [148,149]. In addition, partially or completely unfolded proteins, as well as native proteins containing extensive unstructured elements (longer than 30 aminoacid residues) are substrates of the free 20S CP [150-152]. To this latter group belong especially key regulatory and signaling molecules that are important to many cellular processes [153]. Intrinsically unstructured regions are found in up to 44% of human protein coding genes, making them potential substrates of the 20S [150]. Not much is yet known about the regulation of this pathway, although two proteins, NAD(P)H quinone dehydrogenase 1 (NQO1) [147,154] and protein deglycase DJ-1 [155], have been implicated in 20S regulation upon oxidative stress [155]. The Ub-independent pathway appears to be a critical part of proteostasis control, especially upon oxidative stress, whereas under basal conditions 26S-mediated degradation probably constitutes the majority of proteasomal degradation [156]. A majority of proteins that are degraded through the Ub-independent pathway belong to the intrinsically disordered proteins (IDPs) family [157]. Ub-independent degradation is an important complementary pathway to the Ub-dependent degradation, critical for the removal of damaged and unfolded proteins [145].

1.4.2. Ubiquitin-dependent protein degradation

19S goes through at least four (s1-s4) conformational states [158] during the degradation sequence, reflecting the functional cycle of the proteasome [159].

Substrate degradation consists of the following: 1) initial reversible ATP-activated binding (with Rpn1, Rpn10, Rpn13) [50,160,161]; 2) committing to degradation, which requires an unfolded domain on the substrate and ATP hydrolysis [160]; 3) substrate deubiquitination by Rpn11 (and/or USP14 and UCHL5) [123]; 4) unfolding of the substrate, and its translocation into the CP core for degradation [34,162,163]. It is still somewhat ambiguous what constitutes an appropriate unfolded region to promote effective proteasomal degradation. Research suggests that the region should display minimum flexibility, and contain at least 30 amino acids of varying identity with a bias towards hydrophobic residues [164,165]. Sequences of high repetition show resistance to proteasomal degradation [166], and accumulation of that kind of proteins is linked to many proteotoxic diseases, *e.g.* Alzheimer's or Parkinson's disease [167,168]. The location of the unfolded region in relation to the placement of the Ub-tag(s) potentially influences the importance of the region in ultimately deciding substrate fate [28].

While a protein resides on the proteasome, other competing processes may affect its fate. For example, proteasome-associated DUBs can rescue substrates from degradation after their initial binding to the proteasome [169]. Once a substrate is deubiquitinated, it no longer has a tether at the proteasome, and may escape degradation altogether [15]. To prevent this, the DUB-activity of Rpn11 is coupled to ATP hydrolysis [135], and it acts late in the catalytic cycle, after ATPase engagement, and proper alignment of the substrate for entry into the core [170-173]. The 19S endogenous ubiquitin receptors and proteasome-bound shuttling factors, which include DNA damage-inducible 1 (Ddi1), ubiquitin-domain containing dual-specificity protein kinase 2 (Dsk2) and radiation sensitivity abnormal 23 (Rad23), presumably facilitate testing out different conformations of the substrate, until it is favorably aligned for translocation into the core [15].

1.5. Proteasome modulation

Conventionally, the proteasome has been understood to be a static machine that faithfully degrades substrates from the moment it is assembled until it is ultimately destroyed, with little variation in its function or activity. Conversely, many studies in recent years have demonstrated that the UPS and the proteasome

in particular are regulated in various ways, from orchestrated changes in overall proteasome abundance, proteasome assembly (or disassembly) rate, post-translational modifications (PTMs) of different subunits, to changes in proteasome location and recruitment [9].

1.5.1. Transcriptional regulation

Modulating proteasome abundance through transcriptional events is a significant mechanism regulating the UPS. Following proteasome inhibition or proteotoxic stress, proteasome amounts are quickly increased, requiring the operation of master regulators of proteasome transcription [15]. Two of these, transducer of regulated cAMP response element-binding protein 1 (TORC1) and Extracellular-Signal-Regulated Kinase 5 (ERK5)/mitogen-activated protein kinase 1 (MPK1) are especially important for this process [174]. These two maintain proteasome homeostasis, by reversibly promoting the expression of proteasome subunits and RP assembly chaperones in response to acute stress [174]. On a more general level, proteasome subunit expression is regulated by several transcription factors, including nuclear factor Y (NF-Y), nuclear respiratory factor 1 and 2 (Nrf1 and Nrf2) and their *C. elegans* orthologue skinhead transcription factor 1 (SKN-1), Heatshock factor 2 (HSF2), Rpn4 (in yeast), and abnormal dauer formation 16/forkhead box O (DAF-16/FOXO) [60,175,176]. Subsequently, almost all proteasome subunit promoters have binding sites for these transcription factors, and multiple binding sites for FOXO [60]. They work separately and in concert, along with various other effectors in modulating subunit expression, both following stress and under basal conditions (reviewed extensively in [60]). For example, in yeast stress induces Rpn4 regulated subunit transcription and the expression of proteasome assembly chaperones [175,176]. These transcription factors are also themselves proteasomal substrates, thus forming negative feedback loops with the proteasome [177].

1.5.2. Proteasome assembly and disassembly

The multifaceted proteasome assembly is steered by both intrinsic structural features of the subunits, as well as by dedicated assembly factors [178]. The proteasome is constructed in several parallel and successive steps into a catalytically active complex, facilitated by specific chaperones and maturation factors [9]. Proteasome-assembling chaperones 1-4 form two main chaperone

complexes, PAC1•PAC2 and PAC3•PAC4, which guide the formation of the α-ring [9]. β-ring assembly is in turn mediated by another chaperone, proteasome maturation protein (POMP) [9,179]. POMP additionally localizes the forming proteasome complex to the endoplasmic reticulum (ER), where majority of mammalian proteasomes are constructed [9,179]. The β7 subunit is added as a last step, creating a half proteasome (15S) [9]. The incorporation of β7 prompts the dimerization of two 15S complexes, and at the same time, the β-subunits undergo autocatalytic cleavage, becoming activated [180,181]. However, not all proteasomes are built equal: in the yeast *S. cerevisiae* a second copy of α4 subunit can replace α3, with no apparent adverse effects for the organism [182,183]. α4-α4 proteasomes have also been detected in mammalian cells, which could indicate that this alternative assembly process is evolutionarily conserved, though it has not yet any known function [184].

The two 19S sub-complexes, the lid and the base, are constructed separately [9]. Base-specific chaperones herd the six AAA^+ ATPases Rpt1-6 into forming a ring by binding with distinct subunits, inhibiting at the same time 19S DUB and ATPase activity [30,181]. The construction of the lid sub-complex is thought to proceed in several steps [9], completed by the addition of Rpn12 [30,185]. This triggers a conformational change, enabling the lid and the base to merge together through their mutual association with Rpn10 [30,185]. Proteasome assembly may also be organized differently, depending on prevailing conditions: for example in yeast a stress-induced proteasome assembly chaperone ATPase dedicated chaperone of 17 kDa (Adc17) facilitates 19S assembly following the increased need for more proteasomes [186].

In contrast, the process of proteasome disassembly is poorly understood. The proteasome is known to be for the most part a relatively stable complex, but it is sometimes disassembled, or even fully degraded in response to inhibition and different types of stress. 19S subunits Rpt5, Rpn2 and Rpn10 can be cleaved by Caspase-3, thereby dissociating the lid from the base and obstructing ubiquitin-mediated degradation [187]. Yeast has a specific pathway for the removal of non-functional proteasome subunits, which are then degraded by the proteasome [188]. Further, entire proteasome complexes can be directed to degradation in the lysosome [189,190]. Oxidative stress also induces the dissociation of 26S into its respective sub-complexes; a process that is in part mediated by Heatshock protein 70 (Hsp70) [191].

1.5.3. Post-translational modifications

Multiple post-translational modifications (PTMs) are present on the proteasome at any given time. Proteomic analysis in yeast has identified more than 345 different PTMs, belonging to 11 types [192]. The same site may also host more than one type of modification [193]. Subunits are altered through various post-translational modification mechanisms, such as phosphorylation, acetylation, myristoylation, ubiquitination, O-linked N-acetyl-glucosamine (O-GlcNAc) modification, S-glutathination and oxidation [194,195]. **Table 2** lists proteasomal PTMs with a known target and an identified effect on the proteasome. Several hundred phosphorylation sites alone have been detected, present on every proteasome subunit [196]. As a consequence, the proteasome is reversibly phosphorylated in variety of ways, as environmental cues both from within and without the cell result in fine-tuning of its function [196]. 20S and the 19S base seem to be more prone to phosphorylation than the lid subcomplex [196]. Subunit phosphorylation is also necessary for their incorporation into the proteasome [197]. Interestingly, though many kinases have been identified in proteasome phosphorylation, only one proteasome-associated phosphatase has thus far been characterized: nuclear protein ubiquitin-like containing CTD phosphatase 1 (UBLCP1), which negatively regulates proteasome function in the nucleus, promoting disassociation of the 26S into free 20S and 19S complexes [198].

Ubiquitination of proteasome subunits is also an important post-translational modification. In yeast, Rpn10 monoubiquitination reduces substrate binding [199]. A similar effect was observed in mammalian proteasomes following proteasome inhibition, where ubiquitination of ADRM1(Rpn13) decreased substrate binding, and caused a 80%-90% drop in the processing of model ubiquitinated substrates [200]. Post-translational modifications on the proteasome are highly dynamic, depending on *e.g.* cellular localization of the complex, and modulators present in its immediate area. The various PTM methods increase proteasome plasticity, and the ability of the UPS to respond to different stress conditions.

Table 2. Post-translational modifications on the proteasome with a known target and effect.

Target	Modification	Effect
20S	phosphorylation	↑↓ proteasome activity
20S	poly-ADP ribosylation	↑ nuclear proteasome activity and histone degradation
α4	phosphorylation	↓ proteasome activity
α5	*S*-gluthatiolation	20S gate opening, proteasome activity
α6, β3, β6, β7	acetylation	↑ proteasome activity
α7	phosphorylation	↓ substrate affinity, 26S stability
19S	phosphorylation	↑ ATPase activity
Rpn2	phosphorylation	↓ proteasome activity
Rpn6	phosphorylation	↑ proteasome activity
Rpn10	ubiquitination	↓ substrate binding
Rpn13	ubiquitination	↓ substrate binding
Rpt2	*N*-myristoylation	nuclear proteasome localization
Rpt2	*O*-GlcNAc	↓ proteasome activity
Rpt6	phosphorylation	↑ proteasome activity, ↓ proteasome assembly

Table modified from Livneh et al., 2016. [9]

1.5.4. Proteasome-interacting proteins

Multiple proteasome-interacting proteins (PIPs) have been identified, but their roles on the proteasome remain for the most part unclear, or even contradictory. For example, PI31 has been described to inhibit the proteasome *in vitro* [201], but not *in vivo* [202]. Other studies concluded that PI31 preferentially associates with the canonical 20S [81], and induces dissociation of 20S from its activators [201,203]. Conversely, it may also only be a regulator of the immunoproteasome [202]. Another study reported that in the fruit fly *Drosophila melanogaster*, PI31 is a positive regulator of 26S and a negative regulator of free 20S [204]. Yet another study described PI31 having no effect on either proteasome content or function, and that any observed influence could be proteasome type or cellular context specific [205]. Taken together, the affiliation of PI31 with the proteasome is ambiguous. Another protein, extracellular mutant protein-29 (Ecm29) is also known to associate with the proteasome [206]. In yeast, it directly inhibits proteasome activity [207,208], and regulates proteasome assembly [209]. Ecm29 also induces dissociation of the proteasome into 19S and 20S sub-complexes in response to oxidative stress [210-212]. In addition, shuttling factors (*e.g.* Rad23, Ddi1and Dsk2; see **Figure 3**) also associate with the proteasome. They bind to Rpn1, Rpn10 and Rpn13 subunits of the 19S through their UBL-domains, and to ubiquitinated substrates through their UBD-domains, delivering them to the proteasome for degradation [45]. Thus, several proteins associate with the proteasome, sometimes transiently, possessing regulatory functions that can affect *e.g.* proteasome activity or assembly. Further studies are needed to identify other prospective proteasome binding partners, and to discern their varying roles on the complex.

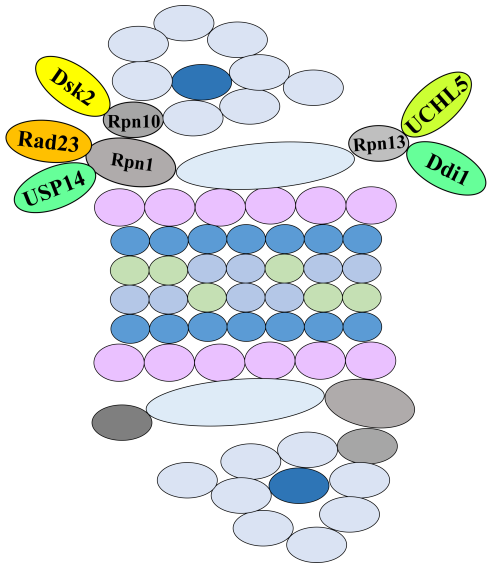

Figure 3. Shuttling factors and DUBs associating with the proteasome. Proteasome subunits Rpn1, Rpn10 and Rpn13 serve as anchor points. Image modified from *Darcy et al., 2015.* [26]

1.5.5. Proteasome-associated deubiquitinating enzymes

A special subclass of deubiquitinases is formed by the proteasome-associated DUBs in charge of substrate deubiquitination, prior to its unfolding and degradation by the proteasome. Ubiquitin bound to the substrate can impede substrate entry, and substrate-free Ub-chains can potentially become stuck on the proteasome. Proteasome-associated DUBs perform several essential functions, both facilitating and inhibiting substrate degradation, all the while maintaining proteasome functionality [134,135]. Most eukaryotic organisms have three major proteasome-associated DUBs: POH1/Rpn11 [134,135], USP14/Ubp6 [46] and UCHL5/Uch37 [47]. Rpn11 is a metalloprotease, while USP14 and UCHL5 are both cysteine proteases [122]. The endopeptidase activity of Rpn11 requires ATP-hydrolysis and intact proteasomes [134,135], while UCHL5 (and potentially USP14) is an ATP-independent exopeptidase [122]. *S. cerevisiae* proteasomes lack UCHL5, and only a small constituent of both mammalian and yeast proteasomes contain USP14/Ubp6 [213-215]. While Rpn11 is a stoichiometric subunit of the proteasome, UCHL5 and USP14 both reversibly associate with the 19S base (see **Figure 3**). Their DUB-activity is increased, or in the case of UCHL5 activated, with proteasome association [26]. USP14 reversibly associates with Rpn1, which increases its DUB-activity 1000-fold, probably through conformational changes that make the catalytic site of USP14 accessible [46,216]. Other DUBs have also been described to occasionally associate with the proteasome, including Usp5, Usp7, Usp9x, Usp13, Usp15 and Usp38, but their possible role in context with the proteasome is not well understood [200,217].

Substrate affinity of the proteasome-associated DUBs depends on position, length, and number of the Ub-chains present on the substrate [28]. These three DUBs are proposed to function collaboratively, and in specific order [28]. UCHL5 and USP14 most likely act before commitment to proteolysis, and Rpn11 during substrate translocation [28]; in a simplified view, Rpn11 promotes degradation [218,219], while USP14 and UCHL5 antagonize it [117,218-220]. Under normal circumstances, cells possess a large pool of free ubiquitin (~20 μM) [221]. It is maintained by the proteasome-associated DUBs; deletion of USP14/Ubp6 alone leads to difficulties in ubiquitin recycling, limiting proteolysis and growth potential [222,223]. Rpn11 is crucial for cell survival in yeast and metazoan systems [224,225], and essential for 19S structure and

activity [225,226]. Rpn11 cleaves an Ub-chain off close to its base, releasing the whole chain at once [28]. Inactivation of Rpn11 traps substrates on the proteasome [227]. In yeast, Ubp6 is not necessary for survival, although it has an important role in the context of metabolic stress [228]. Mammalian proteasomes suffer no structural modifications, and there is no difference in the accumulation of polyubiquitinated substrates after knockdown of USP14 [220]. Interestingly, majority of USP14 proteins appear not to be associated with the proteasome, suggesting that it has roles beyond 26S [220]. Dual inhibition of USP14 and UCHL5 impedes cell growth and degradation, causing accumulation of polyubiquitinated substrates in human cancer cell lines [226]. Conversely, RNAi of either DUB alone causes a reversed phenotype [220].

1.5.5.1. UCHL5/UBH-4

This thesis focuses on UCHL5/Uch37, and its *C. elegans* orthologue, ubiquitin C-terminal hydrolase 4 (UBH-4). The apparent monomeric molecular size of UCHL5 is 37 kDa [229], and it is 329 amino acids long [230]. UCHL5 structure and function are well conserved throughout evolution [230]. Human UCHL5 presents as multiple different splice variants [231]; however, virtually nothing is known about their potential different functions or expression profiles. Though *S. cerevisiae* has no known orthologue of UCHL5 [10], *S. pombe* has a seemingly redundant orthologue, ubiquitin C-terminal hydrolase (Uch2) [232]. UCHL5 association with the proteasome is reversible [52,230,233,234], and it also associates with the INO80 chromatin remodeling complex, although its role there remains obscure [59,235-238].

A central six-stranded β-sheet flanked from each side by α-helixes composes the catalytic domain of UCHL5 [133]. The C-terminal KEKE-motif (a domain enriched in alternating lysine (K) and glutamic acid (E) residues) extension of UCHL5 binds to both Rpn13 and NFRKB/INO80G-subunit through their DEUBiquitinase ADaptor (DEUBAD) domains [52,230,233,235]. UCHL5 is also linked to Rpn2 via Rpn13 [219]. When not bound by INO80 or the proteasome, UCHL5 is DUB inactive, and exists as a mixture of different oligomeric states in the cytosol, where two UCHL5 active sites sterically obstruct substrate access [229]. Binding to Rpn13 induces conformational changes that activate UCHL5 DUB activity [52,230,233]. In contrast, association with NFRKB is highly DUB inhibitory [229]. Transient incubation with Rpn13 or

UCHL5-lacking 26S restored UCHL5 DUB activity, without disassociating UCHL5 from the INO80 complex [235,239,240]. This activation was abrogated, however, when Rpn13 was removed. UCHL5 can trim entire polyubiquitin chains at once, but in the context of the proteasome it removes ubiquitin one molecule at a time from the distal end of the chain [219], cleaving both Lys48- and Lys63-linked chains [117]. UCHL5 is thought to save poorly or ineptly ubiquitinated substrates from degradation [219,220,241]. UCHL5 also exhibits substrate specificity, *e.g.* by promoting the degradation of nitric oxide synthase and Iκβ-α [242]. This suggests that UCHL5 has a dual role in protein turnover, promoting the degradation of some substrates, and inhibiting the degradation of others [26].

UCHL5 has been implicated in Alzheimer's disease [243], and its deletion causes embryonic lethality in mice [244]. Both Rpn13 and UCHL5 are essential for cell cycle progression, as deletion of either gene caused G0/G1 stalling *in vitro* [238]. High levels of UCHL5 have been reported in pulmonary fibrosis, where UCHL5 deubiquitinates the transcription factors Mothers against decapentaplegic homolog 2 (SMAD2) and SMAD3, promoting transforming growth factor-β (TGF-β) stabilization and the expression of profibrotic proteins (*e.g.* fibronectin) [245,246]. Concurrently, UCHL5 functions as an agonist of the SMAD specific E3 Ubiquitin protein ligase 2 (SMURF2), following UCHL5 recruitment by SMAD7 into the TGF-β inhibitory SMAD7-SMURF2 complex, in this manner stabilizing TGF-β from two fronts [136,247-251]. Several distinct roles for the UCHL5 on the proteasome have been suggested, from trimming poorly ubiquitinated proteasome substrates [219], relieving ubiquitination status of proteasome subunits [252] to blocking substrate access to the proteasome [252]. It is possible that the role of UCHL5 on the proteasome is a dynamic mixture of these and other functions not yet identified.

1.6. The ubiquitin-proteasome system in the regulation of proteostasis in aging

The inevitable aging process affects effectively all cellular pathways and processes in most organisms, except for biologically immortal creatures such as *Hydra* [253]. Aged cells must content with an increased amount in misfolded or surplus proteins, resulting in elevated levels of proteotoxic stress [254]. This is often thought to be accompanied with a collapse in proteostasis, in part caused

by a decrease in proteasome activity [255]. Several signaling pathways regulate aging, often with a regulatory role in maintenance of proteostasis [256,257]. The best characterized of these pathways is Insulin/IGF-1 signaling (IIS). Reduced IIS has been shown to delay the onset of many age-related diseases [254], extend lifespan in *C. elegans*, and correlate with longevity in humans [258,259]. In *C. elegans*, an important IIS effector is the sole nematode FOXO homolog DAF-16, which regulates the expression of multiple downstream genes [258]. Reduced IIS phenotype protects from proteotoxicity [260,261] in part by delaying the onset of age-dependent protein aggregation, which is mediated most likely through the modulation of the proteostasis network [262]. Different mechanisms exist to counter the accumulation of misfolded proteins, known collectively as the unfolded protein response (UPR) [8]. These pathways are coupled to the protein degradation machinery of the cell, including the UPS [8,263].

For some time, the consensus has been that protein quality control systems, including UPS activity, decline with age [264]. Notably, UPS dysfunction is present in several age-related neurodegenerative disorders, *e.g.* Alzheimer's [265], Parkinson's [266], or Huntington's disease [267]. Further, a decline in the expression of proteasome subunits has been observed in aging mice [268,269]. Aging-associated decrease in proteasome function has also been perceived in many rat and human tissues [270-274]. Supporting this observation, the promotion of proteasome activity increased lifespan in several model organisms [275-277]. However, the expression of several proteasome subunits was upregulated in aging *C. elegans* [278]. Additionally, following proteotoxic stress, the upregulation of Rpn6 alone was enough to promote proteasome activity, and extend lifespan in *C. elegans* [279]. The observed age-dependent decline in UPS output in multiple systems could be the result of various factors, such as changes in proteasome composition and structure, or availability of other UPS members [194]. Further, changes in proteasome post-translational modifications [280,281] or amassing oxidative damage can also be at fault [282].

However, it is also possible that the two pathways of proteasome mediated protein degradation are affected differently in response to aging. Though in some cases there appears to be a tissue-specific increase in 26S activity [283,284], the ability of 20S to degrade oxidized proteins declines, resulting in the accumulation of harmful oxidized proteins [273,285]. This could cause accumulation of undegradable aggregates, which then stall and trap the proteasomes binding with

them to attempt their degradation [286-288]. Taken together, the details of UPS function and proteasome activity in different cells and tissues of aging organisms remain a fascinating and important question for future research.

1.7. The ubiquitin-proteasome system in cancer

The UPS has been implicated multiple times in malignant neoplastic formation, due in part to its regulation of many cell cycle proteins (e.g. CDK inhibitors and cyclins), and apoptotic factors (e.g. p53 and caspases) [289-294]. Perturbations of the UPS cause aberrant stabilization, or loss of important regulatory proteins, disrupting the normal balance of these molecules, thus contributing to tumorigenesis [295,296]. UPS is often found to be hyper-activated in tumor cells [293,294,297-299], though the increase in activity might not be sufficient to alleviate the increase in accumulating proteasomal substrates [299]. In contrast, CSCs (cancer stem cells) have been reported to exhibit relatively low proteasome activity in some studies [300,301]. This may confer protection from host immune attack, following decreased antigen presentation by these cells [60]. However, though proteasome activity in CSCs may be low, expression of some proteasome subunits is upregulated [60].

Several components of the UPS, in addition to proteasome activity itself, can be affected in cancer. Deregulation of multiple E3s is associated with the development, progression and therapeutic response of several cancer types [98]. Many DUBs are also prominently connected to cancer in a similar manner, including (but not limited to) USP4, USP6, USP8, USP14 and USP28 [302]. In hepatocellular carcinoma, high UCHL5 expression is linked to cancer recurrence [303]. In the same cancer type, UCHL5 was also found to associate with glucose-regulated protein 78 (GRP78) [304], a Hsp70-family chaperone, which is overexpressed in various cancers [305-308]. In esophageal squamous cell carcinoma and epithelial ovarian cancer, high UCHL5 expression correlated with poor prognosis and cancer recurrence [309,310]. Further, high levels of UCHL5 expression have been described in breast, vulva and parathyroid cancers, and increased UCHL5 activity was reported in cervical cancer [311]. Both USP14 and UCHL5 are expressed in high levels in multiple myeloma, and their knockdown is antagonistic for multiple myeloma cancer cells [312].

1.7.1. Targeting the ubiquitin-proteasome system in cancer therapies

Malignant neoplastic cells exhibit amplified sensitivity for proteasomal inhibition [313], due most likely to the increased proliferation and protein turnover in these cells, requiring increased proteasomal degradation [314]. Proteasome inhibitors targeting the catalytic activity of the 20S CP have been used in treatment of cancer since 2003, *e.g.* in multiple myeloma and mantle cell lymphoma [315], and they are currently being investigated for use in solid tumors, though thus far without promising results [316]. As a treatment form, proteasome inhibition is effective, resulting in higher disease free- and overall survival for many patients [317]. However, the use of proteasome inhibitors can also cause many difficult off-target effects, such as severe gastrointestinal (GI) side-effects [317] and peripheral autonomic neuropathy [318]. These inhibitor-linked toxicities can be dose-limiting, ranging from minor to severe [319]. Additionally, treatment resistance is a common problem, especially upon cancer recurrence [320,321]. Refining of combinatory treatments which include proteasome inhibitors might broaden the usefulness of these drugs, also in other cancer types [322].

In view of the multiple problems associated with the use of current proteasome inhibitors, new members of the UPS are being explored for targeted treatment. Tumor cells are subject to constantly changing conditions, and their dynamic plasticity convey a large magnitude of survival potential [98]. This involves *e.g.* the recruitment of many downstream effectors, which is why spatial and temporal deregulation of E3s is considered a viable therapeutic option in many cancers, through the selective inhibition of specific E3s [323]. The aim would be to affect only the target proteins of the E3 involved, with presumably fewer off-target effects [323]. Clinical trials are in progress for evaluating various small molecules that target E1 and E3 enzymes [324-327], and drugs binding and inhibiting E3s are already approved for use [328].

One of the more promising groups of relatively new treatment targets are DUBs, especially the proteasome-associated DUBs Rpn11, USP14 and UCHL5. Deubiquitinases are associated in various cellular processes, which are often found altered in cancer, including cell cycle control and apoptosis [26]. DUBs are able to modulate substrates in a selective and specific manner [329], and their targeting could provide the opportunity to influence only certain aspects of

pathological signaling pathways, instead of globally affecting Ub-mediated protein degradation [330]. DUB inhibitors in development range from pan-enzyme inhibitors to specific inhibitors, targeting just one DUB [330]. Expected effects include *e.g.* accumulation of polyubiquitinated substrates, disruptions in Ub-recycling, and slower polyubiquitin disassembly [331], all of which would be deleterious for cancerous cells, and potentially induce their apoptosis. As some DUBs are substrate specific, targeting just one or few could limit the more serious off-target effects, in a similar manner as when targeting specific E3s, providing increased therapeutic efficacy [332]. In addition, DUBs are presumed to be easier to target than E3s, because E3 enzymes lack universal catalytic residues, unlike DUBs, which are for the majority cysteine proteases [26]. Inhibitors for proteasome-associated DUBs are already in development: for example, inhibition of USP14 with IU1 reduced chain trimming, and increased proteasomal degradation, which could be beneficial additionally in protein aggregate diseases, such as Alzheimer's disease [218]. Another inhibitor, b-AP15, provides dual inhibition of both USP14 and UCHL5, and displays extensive anti-neoplastic activity against solid tumors [333]. b-AP15 has also exhibited promising results in multiple myeloma in animal models [312]. In addition, WP1130-mediated inhibition was shown to target several DUBs, including USP14 and UCHL5, causing accumulation of polyubiquitinated substrates [331].

Lastly, targeting 19S subunits might favor 20S association with alternate activators, such as 11S, which in turn would promote production of anti-tumorigenic peptides for the MHC I complex [60]. In combination with new immune response promoting therapies this might provide beneficial results [334]. UCHL5 binding partner Rpn13 has also emerged as a therapeutic target in different cancers, including Bortezomib (a canonical proteasome inhibitor) resistant multiple myeloma [335]. Further, the immunoproteasome is upregulated in inflamed tissues and cells of the immune system, replacing conventional proteasomes to a high degree [82-84]. Immunoproteasome inhibition in mice exhibited a broader therapeutic window and less side-effects than general proteasome inhibition, and inhibition of the LMP7/β5i subunit shows great promise in colorectal cancer [336]. Considering the variation in the expression of tissue-specific proteasomes and their subunits, using proteasome inhibitors with such narrow range could potentially be used to target distinct diseases, such as autoimmune diseases, with fewer off target-effects.

2. Gastrointestinal cancers

Cancer is a heterogeneous disease, and by its simplest definition, a sickness of the cell cycle; cancer patients suffer unchecked cellular growth and proliferation, with ultimately fatal results. Cancer is often considered a disease of the old, as more than half of cancer patients are over 70 [337]. Aging and cancer are in part caused by similar processes: the time-dependent accumulation of cellular damage [255]. Gastrointestinal cancers constitute a varied group of malignant neoplasms, with an origin in different tissues and organs of the digestive system. They present varying modes of development and degrees of aggressiveness, and they also share some common predisposing environmental factors and genetic traits. Three major gastrointestinal cancers are examined in this thesis: colorectal cancer (CRC), gastric cancer (GC) and pancreatic ductal adenocarcinoma (PDAC).

During carcinogenesis, cellular signaling pathways are repurposed into tumor development following the silencing and/or activation of one or more key molecule(s); either via mutation, or by through some other processes, *e.g.* epigenetic changes [338]. This results in the constitutive activation or suppression of often multiple pathways, all contributing towards harmful neoplastic transformation [339]. Common signaling pathways found altered in CRC, GC and PDAC include, but are not limited to, Wnt-signaling, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK-ERK) pathway, the phosphatidylinositol-3-kinase/protein-kinase B/mechanistic target of Rapamycin (PI(3)K/AKT/mTOR) pathway and tumor protein 53 (TP53) [340], which are all classic oncogenic signaling pathways.

A list of shared genes affected (*e.g.* through mutation, epigenetic modification, amplification) in CRC, GC and PDAC is collected in **Table 3**. Loss of TP53 is common in all GI cancers [341]. Increased β-catenin expression is also frequent, often caused by inactivation of the adenomatous polyposis coli (APC) gene [340]. However, within a given tumor there are generally several clones or families of tumor cells, which have developed in response to the significant selective pressure caused by the abnormal conditions within the tumor itself, and the surrounding stroma [342]. As a result, prognosis, responsiveness to treatment, and emergence of resistance can be erratic and difficult to predict, due to the dissimilar nature in biological and genetic modifications found within the same

tumor [343, 344]. Many cancer cells also exhibit a dynamic range of molecular and phenotypic states during tumor growth and development [345].

Table 3. Common altered genes in CRC, GC and PDAC.

Gene	CRC	GC	PDAC
ALK	X	X	X
AMER1	X	X	
APC	X	X	
ARID1A	X	X	X
ATM	X	X	
BRAF	X	X	
β-CATENIN	X	X	
CDKN2A	X	X	X
DCC	X	X	
E-CADHERIN	X	X	
EGFR	X	X	
HER2	X	X	
KRAS	X	X	X
mTOR	X	X	
NOTCH	X	X	
p16	X	X	X
PI3K	X	X	
PTEN	X	X	
SMAD4	X	X	X
TGF-β	X	X	
TP53	X	X	X
VEGFR	X	X	

X: >5% of cases affected. Alteration frequency checked according to The Cancer Genome Atlas (TCGA) https://portal.gdc.cancer.gov. [339, 346-357]

Metastases spreading out from primary tumors develop similarly in a stepwise process, much like the initial tumorigenesis. Abnormal epithelial cells dedifferentiate through epithelial-to-mesenchymal transition (EMT) to achieve mesenchymal morphology, which allows intravasation into the bloodstream as

circulating tumor cells (CTCs) [358]. In the blood, CTCs can assume mesenchymal or epithelial phenotype, or distinct characteristics of both [359]. However, mesenchymal CTCs almost certainly harbor a higher capacity for metastasis, due to their better ability to extravasate back into tissues [360]. These secondary tumor sites often have a very different mutational load compared to the primary tumor, which again might have a significant effect on treatment responsiveness and prognosis, especially upon cancer recurrence [343]. This should be taken into account in the use of biomarkers, which are usually only sampled at the primary tumor site [349]. While some studies report a high degree of concordance between primary tumors and metastases (at least in CRC), there exists a possibility of complex heterogeneity, in part caused by the differences found in tumor microenvironments (TME) present at distant metastatic sites [361,362].

Accurate cancer classification is required for correct diagnosis and treatment of the patient. Therapy options in GI cancers depend largely on the stage of the disease upon diagnosis, and the need for pre- or postoperative adjuvant therapy should also be assessed on an individual basis [363]. The anatomic site and histologic appearance of the tumor are critical for determining these factors; however, morphologically similar cancers can vary wildly in their course and response to treatment [364]. The globally used TNM Classification of Malignant Tumors (TNM staging; see **APPENDIXES**) considers the type of the original tumor (size, involvement with nearby tissues; T), lymph node-involvement (N) and metastatic status (M). The 8th iteration of the system is recommended for use since the beginning of 2018.

2.1. Colorectal cancer

The large bowel is divided into cecum, colon and rectum, which comprises the final straight part of the gut. Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world, and also third in cancer-related death [365]; of all GI cancers, CRC is the most common [366]. Nearly 20-30% of CRC cases are diagnosed at an advanced stage, and 40-50% of those discovered in the early stages suffer a relapse [348]. 5-year survival rate varies from 75%-90% for stage I disease, to less than 10% in metastatic CRC [348, 367]. Colorectal tumors originate more often on the left side of the gut [366], and different genetic

aberrations are also associated with each side [346]. Based on anatomy and embryonic origin, CRC is divided into distal colon cancer and rectal cancer, both arising from the hindgut, and proximal colon cancer, arising from the midgut [346].

Due to the elevated turnover rate of the intestinal epithelium, it is a major site for colorectal carcinogenesis [368-370]. Tumors often progress slowly, usually forming over a period of 10-15 years [366]. Their development starts with abnormal transformation of gastric epithelium; aberrant crypt foci (AFC) form in the mucosal crypts located in the rectal or colonic inner lining, creating the first precursors to cancer [371]. AFCs progress into adenomatous polyps or adenomas [372,373], although it is postulated that they may themselves directly become cancerous [374,375]. Over 95% of all CRC cases are adenocarcinomas [373], which develop from the mucosal glands lining the gut [373]. Other CRC types include intestinal lymphomas and sarcomas, carcinoid tumors (originating from intestinal hormonal cells) and gastrointestinal stromal tumors (originating from the interstitial cells of Cajal or ICCs) [373].

Akin to all cancers, CRC is a consequence of different factors, including inherited elements and traits acquired during individual lifetime. Genetic factors influence approximately 35% of CRC, both protecting from, or causing cancer development [376]. While most cases of CRC are considered sporadic [377], approximately 5%-6% of CRC patients have an identified inherited predisposition [367,378]. Hereditary colorectal syndromes include *e.g.* familial adenomatous polyposis (FAP; variants include attenuated FAP and familial colorectal polyposis/Gardner's syndrome), juvenile polyposis syndrome (JPS), hereditary nonpolyposis colorectal cancer (HNPCC or Lynch disease), hereditary mixed polyposis, MYH-associated polyposis (MAP), Peutz-Jeghers syndrome (PJS) and serrated polyposis syndrome (SPS) [346,350]. Of these, HNPCC is by far the most frequent, consisting of approximately 2-5% of all CRC cases [346]. Sporadic CRC is thought to progress with successive accumulation of somatic mutations in both tumor suppressor and proto-oncogenes, leading to malignant transformation, where the total accumulation of genetic modifications is important, rather than their specific order [374]. One study identified an average of 80-90 mutated genes per colorectal tumor [379,380], of which 15 were considered true drivers [379].

The three main pathways related to oncogenic development of both hereditary and sporadic CRC are the chromosomal instability (CIN) pathway, the mismatch repair pathway (MMR) and the CpG island methylator phenotype (CIMP), which all have distinct phenotypes, and are linked to specific genetic and molecular signatures [347]. All three can be present separately or in combination in a given tumor, greatly affecting *e.g.* tumor malignancy and other clinical traits, such as treatment response [367,381]. The CIN pathway is best known in association with the well-known adenoma to carcinoma sequence [347,382] and is present in 70% of all CRC [383]. It describes the classical stepwise transformation of regular colonic mucosa to carcinoma, with multiple accumulating mutation events on the way [347]. The MMR pathway is sometimes also called microsatellite instability (MIN/MIS/MSI), which is a phenomenon associated with this pathway. Microsatellites are 1-4 nucleotide long sequences of repetitive noncoding DNA, which are found throughout the genome [347]. DNA mismatch repair (MMR) proteins are a family of genes that repair mutations occurring during the replicative cycle, protecting microsatellite stability [347]. Both genetic and epigenetic silencing of these genes leads to accumulating mutations, causing MSI and hastening oncogenic transformation [384]. CpG islands are CG-rich sections of DNA present in approximately half of all genetic promoters [382]. The CIMP pathway is characterized by the presence of ubiquitous CpG island methylation, causing aberrant gene silencing [385].

Genes altered in CRC are listed in **Table 3**. The best characterized genetic defect associated with CRC are APC mutations, the loss of which seems to be an early event in CRC tumorigenesis [386]. Approximately 80% of all sporadic CRC tumors have these mutations [387]. APC is a member of the Wnt pathway, which regulates the cytoplasmic levels of β-catenin [387]. Increased β-catenin levels induce expression of a proliferation regulator, MYC [388]. Together, APC, β-catenin and MYC are involved in maintaining cell integrity, and loss of APC often leads to decreased cell-to-cell adhesion, and an increase in cell migration and metastatic potential [388]. The mutation of V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) is common both in the primary tumor and metastatic tumor sites [389]. RAS mutations (BRAF/KRAS/NRAS) are often accompanied by phosphorylation of ERKs, and the activation of the ERK/MAPK pathway [390]. RAS signaling is mediated also through PI(3)K [391], resulting in promotion of cell survival and growth [348]. In contrast, the PI(3)K inhibitor phosphatase and tension homolog (PTEN) is inactivated in 10% of CRC cases

[348]. The regulatory oncogenes MYC and CDK8 are rarely mutated in CRC, but their amplification is present in approximately 10%-25% of tumors [392,393], and alterations in MYC activity are present in approximately 95% of CRC [348]. The most crucial altered CRC genetic pathways are Wnt, MAPK, PI(3)K, TGF-β and TP53 [348]. Of these, most often affected is the Wnt pathway: constitutive activation is found in approximately 90% of studied tumors [348, 394]. Only 5% of adenomas harbor *p53* mutations, but they are more common in malignant polyps (50%) and metastatic CRC (75%) [395].

Though the genetic background of the patient plays a significant role, many environmental traits predispose patients to CRC. Known risk factors include obesity, lack of exercise, use of alcohol and tobacco, as well as *e.g.* fat-rich, high-meat and fiber-deficient diet [373,396-399]. In addition, high levels of insulin and chronic intestinal inflammation (*e.g.* Crohn's disease, ulcerative colitis) have been linked with higher risk of CRC [373,400]. Globally, patients over 50 years old are more likely to develop CRC [401], and men are two times more likely to fall ill than women [369]. In contrast, diet rich in fiber [402] or fish (particularly consumption of fish oil) [403], high intake of vitamin D or calcium [404], and regular aspirin use at least twice per week [405] all reduce risk of CRC. Interactions between environmental and genetic factors converge to affect individual probability to develop CRC.

The Dukes' system for CRC staging was developed in 1932 by the British pathologist Cuthbert Dukes [406]. Originally used only for colonic tumors, Dukes' has been later modified, thereby creating the Astler-Coller modification (MAC), Tunrbull modification (1967) and the Australian Clinico-Pathological Staging (ACPS) modification [407-409]. However, the Dukes' system has been widely replaced by the TNM staging system in clinical praxis (described in **APPENDIX 1A** and **1B**). Dukes A corresponds to stage I, Dukes B to stage II, Dukes C to stage III and Dukes D to stage IV of the current TNM system (**APPENDIX 1A**). Characterization of CRC based on molecular subtypes rather than histology has been attempted by different groups [392,393,410-413], classifying 3-6 different molecular CRC subtypes depending on the study. At least in the case of *Sadanandam et al*., the characterized subtypes seemed to have both prognostic and therapeutic value [411]. The Colorectal Cancer Subtyping Consortium (CRCSC) has also attempted molecular classification [414]. Their categories include four different molecular CRC subtypes (CMS1-4), and a fifth

less clearly defined subgroup, all displaying similarities in molecular, clinical and pathological traits [414]. Despite these advancements, some CRC tumors still defy classification, and molecular CRC classification methods require improvement before they can be applied in a clinical setting. More precise molecular subgrouping could clarify therapy options, and provide more accurate prognostic information [346, 348].

CRC is unfortunately often symptomless until tumors have reached a large size [366]. Common symptoms connected to CRC include rectal bleeding, changes in bowel movements and abdominal pain, but they are also associated with other gastrointestinal conditions, and their usefulness in diagnosing CRC is estimated to be less than 10% [415]. Treatment options involve surgery, chemotherapy, combined radio-chemotherapy and more recently, monoclonal antibody-based therapies directed at the vascular endothelial growth factors (VEGFs) or epithelial growth factor receptor (EGFR) [336]. While adjuvant treatment offers no survival benefit for stage I patients, a well-chosen treatment may improve both life quality and length of stage III patients and stage II patients presenting adverse prognostic factors (such as blood vessel- or perineural invasion) [350]. In recent decades, improvements have also been made in the treatment of patients with metastatic cancer, but in general terms CRC remains a difficult and potentially fatal disease.

2.2. Gastric cancer

The stomach is divided into fundus, corpus (body) and pyloric antrum. Gastric cancers (gastric carcinomas, GCs) are globally the fifth most common cancer with third highest cancer-related mortality [354,416,417]. With a 5-year survival rate of less than 30% [418-420], GC is also often highly invasive and metastatic [421-423]. The biology of gastric cancer is mixed, evolving from various epigenetic and genetic alterations. Similarly to CRC, GC develops with the progressive accumulation of adverse gene modifications in gastric epithelial cells that ultimately contribute to malignant neoplastic transformation [424,425]. Chronic gastric infection (gastritis) coupled with intestinal metaplasia (IM) are crucial in early GC pathogenesis [426]. Hereditary forms of GC are rare (only 1-3% of cases), and they fall predominantly into three types: gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), familial

intestinal gastric cancer (FIGC) and hereditary diffuse gastric cancer (HDGC) [426].

Classically, gastric adenocarcinomas are divided according to their histological subtype using the Laurén classification into intestinal or diffuse tumors [354,427-429]. The intestinal-type adenocarcinomas typically develop glands with varying degree of differentiation, while diffuse gastric cancer carcinomas lack cohesiveness, being composed of single cells or small groups of neoplastic cells scattered inside the gastrine wall [354]. **Table 3** lists genetic alterations found in GC. The genomic instability pathways relevant in the development of CRC (CIN, MSI and CIMP) are equally important in GC carcinogenesis [430]. Other pathways that are often mutated include chromatin remodeling associated genes [431,432], cell motility/cytoskeleton associated genes [433], Wnt signaling [340] and receptor tyrosine kinases [295,431,432,434-437]. The cell-adhesion molecule E-cadherin is more often mutated in diffuse GC, including hereditary diffuse GC [351].

Perhaps the most well-known environmental risk factor associated with gastric cancer is chronic *Helicobacteria pylori*-infection [363,429]. Some studies suggest as high a risk as 75% of developing GC linked with *H. pylori* [438-440]. Similarly, the Epstein-Barr virus (EBV) is linked to GC [441]. Increased salt intake, smoked or spoiled food, nitrates and secondary amines increase risk of gastric cancer [363,442]. Other high risk factors include smoking and heavy alcohol use [363]. Conversely, substantial intake of raw vegetables, fruits, vitamins A and C, calcium and antioxidants reduce gastric cancer risk [443].

The TNM-staging system is in common use for prognostic purposes in the clinic (see **APPENDIX 2A** and **2B**). Attempts have been made to develop other classification systems, which would be more accurate in the prognostic setting. World Health Organization (WHO) has introduced a new type of classification with the following subgroups: tubular, papillary, mucinous and poorly cohesive/signet ring [444]. The cancer genome atlas (TCGA) research project categorized four major molecular subtypes of GC tumors: Epstein-Barr virus (EBV)-positive, microsatellite instability (MSI), genomic stability (GS) and chromosomal instability (CIN) [431]. A similar study by the Asian Cancer Research Group (ACRG) divided gastric cancer into four different subgroups: MSI, microsatellite stable (MSS)/epithelial-to-mesenchyme (EMT)-positive,

MSS/TP53^{+} and MSS/TP53^{-} [432]. In comparison to the TCGA grouping, these four subgroups also presented significant survival differences, as well as distinct clinical properties [432]. Despite these developments, reliable treatment options based on these molecular subtypes are not yet available [351].

Most gastric cancer patients are diagnosed at an advanced (stage III-IV) stage of cancer, and they are often deemed unresectable by this point [445]. In metastatic GC, systemic chemotherapy is currently the only available treatment option [446]. Regrettably, inherent and acquired treatment resistance is a large problem, and most patients die within a year from first diagnosis [447,448]. Gastric cancer symptoms can easily masquerade as other nonthreatening gastric conditions, but actual symptoms include anorexia, dysphagia, vomiting, gastrointestinal blood loss and weight loss [449]. For these reasons, the prognosis for gastric cancer patients in the current treatment landscape remains poor [450].

2.3. Pancreatic ductal adenocarcinoma

Over 90% of all pancreatic cancers are classified as pancreatic ductal adenocarcinoma (PDAC) [451]. Even with successful resection in early PDAC, lymph node-negative patients have a 5-year survival of 25%-30%, and lymph node-positive as low as 10% [452]. Further, advanced PDAC cases consist 4% of global cancer related deaths [357], with all patients exhibiting a 5-year survival of 8% [420], and only 1% for metastatic cases [357]. Majority of genetic or metabolic changes underlying the development of PDAC are sporadic [357]. Hereditary mutation load explains approximately 5-10% of PDAC, although the inherited component has not been identified in most cases [453,454]. Inherited PDAC occurs in three distinct forms: hereditary tumor predisposition syndromes, and hereditary pancreatic and familiar pancreatic cancer [453,455]. Genetic factors increasing incidence are family history of pancreatic cancer [456], familial atypical multiple melanoma and mole syndrome (FAMM), familial breast cancer, Lynch disease, PJS and Fanconi anemia [456-458]. Though there is high degree of heterogeneity within tumors, primary tumors appear to share largely overlapping genomic traits with metastases, even if they arise from distinct subclones of the original tumor [459]. PDAC has also a high metastasis rate, through pre-metastatic niche-formation usually already early in tumorigenesis [339].

Table 3 contains a list of gene alterations present in PDAC. Gene mutations include the oncogene KRAS, found in chronic pancreatitis [460] and in approximately 90% of PDAC cases, occurring often early in tumorigenesis [461-463]. The tumor suppressor *p16*, in connection with CDK inactivation, is mutated in almost 95% of patients, causing uncontrolled proliferation [461,462,464]. SMAD4 mutations are present in almost 50% of tumors, conveying abnormal TGF-β signaling [461,462]. SMAD4 loss is additionally connected to significant metastasis [356]. 75% of pancreatic cancers are TP53 mutated [461-463]. Key pathways altered in PDAC malignancy include EFGR signaling [465-467], NOTCH signaling [468,469], Hedgehog signaling [470, 471], Wnt signaling [472,473] and NFκβ signaling [474-476], as well as various cytokines, growth factors and associated signaling [477-480].

Additionally, the surrounding stroma and the tumor microenvironment are very important for the onset and development of PDAC, both at the site of the primary tumor, but also in the forming of eventual metastases [481-484]. Excessive desmoplastic reaction induces proliferation of fibroblasts and production of extracellular matrix (ECM) in PDAC [339]. Stromal cells, such as active fibroblasts, myofibroblasts and pancreatic stellate cells, all actively contribute to the changing ECM composition, along with other stromal elements, such as multiple growth factors (*e.g.* TGF-β) [485,486]. Despite changes in expression profiles and metabolic signatures, actual genetic changes in the stroma are rare, though epigenetic alterations may be present especially in fibroblasts [487-490]. Conversely, stromal cells are also thought to induce epigenetic chances in pancreatic cancer cells [491]. The stroma not only promotes malignant neoplastic formation, but it may also form a drug delivery barrier, hindering PDAC treatment [357].

Environmental risk factors include smoking and obesity [456,463,492,493], diabetes [494], age over 50 [495], ethnicity [456,492,495,496], male gender [495] and the presence of chronic or inherited pancreatitis [497]. *H. Pylori* infection may increase the risk of pancreatic cancer [456,495,498]. On average, PDAC patients are 71 years old [357]. With only 9% of patients diagnosed with localized disease, majority present with either advanced or metastatic PDAC at the time of diagnosis [357,499-501]. PDAC is difficult to treat for multiple reasons, including often late onset of the disease, associated with other medical co-morbidities [357,499-501]. For example, at least 50% of PDAC patients also

suffer from diabetes [502]. PDAC has often asymptomatic progression, but symptoms may include weight loss, nausea, vomiting, jaundice or abdominal pain [503]; symptom types are connected to the location of the tumor [504].

TNM staging for PDAC is described in **APPENDIX 3A** and **3B**. To improve accuracy of PDAC staging, several groups have attempted to classify PDAC tumors. A recent review by *Fakhri and Lim* [364] described four suggested classification systems for PDAC, which are briefly described here. *Collison et al.* used PDAC gene expression analysis, categorizing four different subgroups of PDAC [505]. Whole-genome sequencing and copy-number variation analysis also divided PDAC into four different groups [506]. Transcriptome analysis discovered yet four more subgroups, of which three overlapped with those characterized by *Collison et al.* [505,507]. With somewhat different approach, Moffitt *et al*. also included the surrounding stroma in their analysis, identifying two tumor- and two stroma-specific PDAC subtypes [506]. However, a comprehensive molecular and genetic staging classification system with prognostic or therapeutic relevance for PDAC still remains to be developed.

As with many other cancer types, surgery is the only potentially curative therapy, but possible for only about 15%-20% of PDAC patients at the time of diagnosis [508,509]. The shape and size of the primary tumor, cancer stage (usually only stages I-II) and the involvement of major local vessels, all play a role in the decision to perform surgery [510]. In resectable PDAC, adjuvant therapy can be systemic therapy, for reducing metastatic load, or chemoradiotherapy, though some studies have disputed the usefulness of adjuvant radiation therapy [355,511,512]. Neoadjuvant chemoradiotherapy is sometimes used, though there is no consensus for its benefits [355,513]. In locally advanced, unresectable pancreatic cancer (stage III), use of radiotherapy may even be questionable, as it has given contradicting results [514,515]. Chemotherapy treatment options remain, but their results are patient dependent and difficult to predict [355]. In metastatic disease (stage IV), patients receive mostly palliative chemotherapy [516,517]. Over 90% of diagnosed patients eventually die from the disease; 70% from extensive metastases, and 35% of massive primary tumors [459]. Therefore, development of biomarkers for early diagnosis and identification of predisposing genes is essential.

2.4. An overview of gastrointestinal biomarkers

Biomarkers are usually divided into four types: diagnostic, predictive, prognostic and therapeutic. Diagnostic markers are used as non-invasive markers to detect early disease phases [354]. A prognostic marker should reflect patient survival, and ideally provide knowledge of treatment outcome, as well as suggest further therapeutic options [518,519]. Predictive biomarkers are used to identify subpopulations of patients who would benefit (or not) from a targeted treatment [520]. Therapeutic markers are themselves targets for therapy [354]. The aim of personalized medicine is to tailor treatment specifically to an individual patient, depending on the unique genetic makeup of their tumor(s). For this reason, there is an urgent need for identifying new biomarkers for the detection, diagnosis, prognostic prediction and evaluation of the viability of therapeutic approaches in all three cancer types studied in this thesis. A list of GI-cancer biomarkers is gathered in **Table 4**.

Common biomarkers include nucleic acids, carbohydrates, proteins, lipids, small metabolites, and whole tumor cells isolated from body fluids [521]. Serum genetic material can also be used for screening for epigenetic changes, including aberrant CIMP phenotype, microRNA profile changes and histone modifications, which are often present early in carcinogenesis [522]. Circulating tumor cells (CTCs) are shed into the bloodstream from solid tumors [523,524], including the precursor cells of distant metastases [525,526]. However, while CTCs are not present in healthy controls, their presence is not invariably cancer related [527]. Prognosticative panels often take into account many and varied biomarkers, which help to subgroup patients, and more easily create targeted therapy curated to individual patient needs [452].

Table 4. Biomarkers in use or in development in CRC, GC and PDAC.

	Biomarker function		
Biomarker	**CRC**	**GC**	**PDAC**
18q loss of heterozygosity (LOH)	PRO		
BRAF	PRO		
Carbohydrate antigen (CA 19-9)	PRO	D/PRO	D/PRO
Cancer stem cells (CSCs)	PRE/PRO	D/T	
Carbohydrate antigen (CA 125)		D/PRO	PRO
Carbohydrate antigen (CA 50)		D/PRO	
Carbohydrate antigen (CA 72-4)		D/PRO	
Carcinoembryonic antigen (CEA)	PRO	D	D/PRO
CDH1 (E-cadherin)		PRE	
Chromosomal instability (CIN)	PRO		
Circulating tumor cells (CTCs)	PRE/PRO		D/PRO
COX-2	T/PRO		
CpG islan methylator phenotype (CIMP)	PRO		
Cytotoxi T-lymphosyte antigen 4 (CTLA4)	T		
Dihydropyrimidine dehydrogenase (DPD)	PRO		
EGFR	T	PRO	
ERCC1	PRO		
FGFRs		T	
Gastric carcinoma-associated antigen MG7-Ag		D	
Glurathione S-transferasen P1 (GSTP1)	PRO		
HER2		PRE/PRO	
KRAS	PRE/PRO		
long non-coding RNAs		PRO?	
MET		PRE/PRO	
Methylenetetrahydrofolate reductase (MTHFR)	PRO		
microRNAs		D?	D/PRE/PRO
Microsatellite instability (MSI)	PRE/PRO	PRO	
MLH1	PRO		
mTOR		PRO	
NRAS	PRE		
PEPSINOGENS		D	
PIK3CA	PRE		
Programmed cell death protein 1 (PD1)	T	T	
PTEN	PRE		
Thymidine phosphorylase (TP)	PRO		
Thymidylate syhthetase (TS)	PRE		
TIMP	PRO		
TP53	PRE	D/PRO	
UGT1A1	PRO		
VEGF/VEGFR1/KDR	PRO		
VEGF/VEGFR2	T/PRO		
hENTI			D
cell free DNA (cfDNA)/ circulating tumor DNA (ctDNA)			D

D = diagnostic, PRE = predictive, PRO = prognostic, T = therapeutic;
[354,366,452,521,527-529]

Recent years have seen an increase in the development of biomarkers for many GI cancers. Microsatellite instability is present in 10%-15% CRC, and can be used for evaluating disease outcome: patients with MSI-High phenotype have a better prognosis [530]. KRAS mutations exist in 20%-40% of CRC [531], and the presence of WT KRAS is used as a prognostic marker or to assess therapy effectiveness [532-534]. The number of lymphocytes present in tumor periphery and in its core are used in determining Immunoscore, a type of prognostic index for CRC patients, and a similar method is in development for gastric cancer [535,536]. A higher density of lymphocytes translates to a higher score and a better prognosis (reviewed in [536]). As a prognostic determinant, the Immunoscore was found to be more accurate in stage I-III patients than the classical TNM staging [537], and its incorporation into the TNM staging system in CRC is being considered [536,538]. However, few predictive markers are available to help select therapeutic strategies in CRC [366]. A further problem in these studies rises from contradictory results, small cohort sizes and inconsistencies in study methods between groups [366].

Extensively studied biomarkers in gastric cancer include HER-2, *E*-cadherin, FGFR, EGFR, mTOR, HGFR, MET, PD-L1 and TP53 [354]. Emerging biomarkers in GC include microRNAs, long noncoding RNAs (lncRNA) and matrix metalloproteinases (MMPs) [354]. With the exception of HER-2, there are no established predictive biomarkers, and most GC patients do not yet benefit from directed molecular therapies [354]. Sialyl Lewis carbohydrate antigen 19-9 (CA19-9) is a commonly used serum biomarker for PDAC, although it is sometimes also expressed in non-cancerous conditions, and not by all pancreatic cancer patients [527,539]. Additionally, a part of the population (approximately 10%) do not produce this antigen [540-543]. The second most common serum biomarker in use for PDAC detection is carcinoembryonic antigen (CEA), but unlike CA19-9, it is not recommended for use in the US, having low sensitivity and specificity [544]. CA125/*MUC16* transmembrane glycoprotein overexpression is present in many cancers, including PDAC [545,546], and it *e.g.* predicted resectability better than CA19-9 [547]. Circulating exosomes have been suggested as diagnostic or prognostic markers [548], such as using Glypican-1 (GPC1)-positive exosomes as biomarkers for early PDAC, but this research needs further study [549]. Alterations in microRNA (miRNA) profiles are prevalent in PDAC, and candidate miRNAs have been suggested for detection of early PDAC [550]. There are no specific markers for early diagnosis of

pancreatic cancer [339,356], although candidate RNA and protein biomarkers are being tested [339].

Studying protein expression levels from tumor samples is a well-established way to identify new biomarkers that can then be developed for future clinical use. Tissue microarray blocks (TMA) are used to take small samples from the core of a patient tumor sample, which are then treated with immunohistochemistry to assess tissue immunoexpression levels of target gene(s). TMA allows processing of large patient cohorts in a relatively short time, and the study of multiple potential markers from the same tumor sample. When combined with long follow-up period and extensive survival data, markers with prognostic potential can be identified with relative ease. This method only samples a small part of any given tumor, however, and contains little information *e.g.* about the surrounding stroma. This method should therefore be complemented with further molecular studies to confirm a potential biomarker role in cancer, such as use of organoid models derived from patient tumor samples. In conclusion, extensive work is required to identify and develop new, more accurate biomarkers for clinical use.

3. *Caenorhabditis elegans* as a model system in biomedical research

The experiments in the first part of this thesis were performed extensively by taking advantage of the well-established model animal, *Caenorhabditis elegans*. Since the work of Dr. Sydney Brenner in the late 1960s, the small (1 mm), terrestrial roundworm has developed into a favorite tool in many fields of biomedical research, ranging from molecular genetics to large-scale behavioral studies. *C. elegans* is an androdiecious species: individuals are hermaphrodite (XX) or male (X0). *C. elegans* lifespan is relatively short: 3.5 days in 20 °C, comprising four larval stages (L1-L4), followed by a young adult stage, where egg-laying begins. Hermaphrodite reproduction continues for 1-3 days, and the animal will then live a further 1-2 weeks, which has made *C. elegans* a distinct favorite in aging research. During times of stress (*e.g.* limited food, crowding), L1 larvae can develop into an alternate stress-resistant *dauer*-form, which may live several months without nutrition. [553]

Lack of reliable antibodies is one of the few limitations associated with the animal, which is why *C. elegans* is often used together with other, complementing model systems; yet, despite its apparent simplicity, several key characteristics make it an excellent model animal for multiple types of research. The nematodes are easy to handle and cheap to propagate, and strains can be frozen and stored in -80 °C or in liquid nitrogen. *C. elegans* has several different tissues, including neurons, muscle and intestine. The animal has approximately 1000 somatic cells, which follow an invariable and well-recorded pattern of division and development. The well-annotated (Wormbase.org) 100 Mb genome is organized into six pairs of chromosomes, and has approximately 20 000 genes, with thousands of mutants available for researchers. In addition, *C. elegans* has a small mitochondrial genome of 36 genes. Many important signaling pathways and homologous disease genes are evolutionarily conserved, making the animal a good starting point for varying disease-related research [554]. Further, approximately 60%-80% of human genes have *C. elegans* homologues. Alleles are predominantly homozygous, due to repeated selfing of strains. For genetic studies, knockdown of individual genes with RNAi is easy to perform, as plasmid constructs can be introduced into the bacteria fed directly to the animals. *C. elegans* is also transparent, which allows creation of transgenic lines expressing fluorescent marker proteins, *e.g.* for the study of protein expression or turnover, including degradation of proteasomal substrates. [555]

AIMS OF THE STUDY

The aim of my thesis was to find new proteasome regulators, and characterize in detail their effect on the proteasome. As similar studies have been conventionally performed in yeast or by using mammalian cell cultures, the main objective was to gather information *in vivo* in the context of a complex, multicellular organism. Identification of the novel proteasome regulator UCHL5/UBH-4 in the first part of this thesis was then expanded into an extensive project to study this proteasome-associated DUB in gastrointestinal cancers.

More specifically, the aims were:

1. to acquire new information on how the UPS is regulated *in vivo* on molecular- and tissue-specific level, by taking advantage of the well-established model system *C. elegans*, and

2. to investigate the role of the proteasome modulator UCHL5 in different gastrointestinal cancers, by comparing UCHL5 immunoexpression with clinicopathological factors and patient survival in colorectal cancer, gastric cancer and pancreatic ductal adenocarcinoma patient tumor samples.

PATIENTS, MATERIALS AND METHODS

Materials and methods are described in detail in their respective publications. This section provides a summary of the most relevant methods for the thesis.

1. *In vivo* methods used in I

1.1. Nematodes

All *C. elegans* strains were grown under standard laboratory conditions (in 20 °C incubator) on normal growth medium (NGM) agar plates with *Escheria coli* OP50-strain as their food source [556]. N2 strain (Bristol isolate) was used as the wild-type reference strain in all experiments. The *C. elegans* strains used in the thesis are listed in **Table S1** of publication **I**. Plasmid construction for creation of the transgenic lines was performed according to standard cloning techniques, and microinjection was used for the generation of transgenic animals, as described in [557].

1.2. *In vivo* UPS-activity reporters and polyubiquitin reporters

Transgenic *C. elegans* strains used in the thesis are listed in **Table S1** of publication **I**. The generation of the UPS-activity reporter is described in detail elsewhere ([558], **I**). Briefly, for the UPS-activity reporter, N2 and other relevant *C. elegans* mutant strains were injected with a plasmid containing either the tissue-specific short-lived UPS activity reporter (*UbG76V-Dendra2*), which is a proteasomal substrate, or its long-lived control (*Dendra2* alone). These reporters can be irrevocably photoconverted with 405 nm from a green to red fluorescent conformation, and the variation in the red signal as a function of time can then be followed by fluorescent microscopy. The polyubiquitin reporter (*Pvha-6::UIM2-ZsProSensor*) is a green fluorescent protein (ZsG), fused with the two UIM-motifs of 19S Rpn10/S5a subunit, and the C-terminal part of the mouse ornithine decarboxylase (MODC), which directs the protein to be degraded by the proteasome. The UIM-motifs, however, stabilize the reporter in the presence of polyubiquitinated proteins, and the resulting green fluorescent foci can thus be

interpreted to reflect polyubiquitinated protein amount in the cell (validation of the reporter is described in **I**).

1.3. Lifespan and progeny assays

All lifespan assays were performed at 20 °C. Animals were age-synchronized by hypochlorite treatment, which dissolves adults and larvae, but not unhatched eggs. The eggs are left to hatch in M9 (22 mM KH_2PO_4, 42 mM Na_2HPO_4 and 86 mM NaCl in MQ) buffer overnight, and then plated on RNAi feeding plates as L1 larvae (day 1). The animals were checked daily, and were classified as dead, if they failed to react to a tender jab with a platinum worm pick. Animals that had internally hatched offspring, died from extruded gonad, crawled off or disappeared were censored from the total animal count on the day of occurrence. For the progeny counts, age-synchronized animals were plated on RNAi feeding plates as one day old (L1 larvae), picked individually to separate plates as three days old (L4 larvae), and moved daily to fresh plates to avoid crowding. Total viable offspring per animal was then counted. The offspring of animals that had internally hatched offspring, died from extruded gonad, crawled off or disappeared were censored from the experiments.

1.4. Mammalian cell culturing

Human osteosarcoma (U-2 OS) cells were cultured using Dulbecco's modified Eagle's medium including 15% fetal bovine serum (Gibco). FlexiTube GeneSolution for *uchl5* and AllStars Negative Control siRNA with HiPerFect Transfection Reagent were added to the cells for siRNA (small interfering RNA), all acquired from Qiagen. Fugene 6 (Roche) was used to transfect pEGFP-C1-Ataxin3Q28 (Addgene plasmid 22122), pEGFG-C1-Ataxin3Q84 (Addgene plasmid 22123) [559] and UbG76V-GFP (Addgene plasmid 11941) [560] expression vectors. Cells were fixed in 3.5% paraformaldehyde in phosphate buffered saline (PBS). PI(3)K inhibition was performed with 7 h treatment with 20 μM LY-294002 (Enzo Life Sciences).

1.5. Microscopy and image analysis

The transgenic UbG76V-Dendra2 and Dendra2 animals were imaged at four days old (young adult stage). The animals were mounted on a small agarose pad on a glass slide and immobilized using 0.5 mM levamisole diluted in M9 buffer. In between imaging, animals were allowed to recover freely on feeding plates. A motorized Zeiss Axio Observer Z1 inverted microscope with LSM 5 Live line-scanner was used for imaging. LSM AIM software Rel. 4.2. was used. A 63x 1.4 NA plan-apochromat objective was used for acquiring images. Zeiss LSM image Examiner version 4.2 was utilized for analyzing the fluorescence intensities of the images. Transgenic polyubiquitin reporter (*Pvha-6::UIM2-ZsProSensor*) animals were imaged as six days old, and strains carrying transcriptional reporters were imaged as four days old (young adult stage). These strains were imaged with Axioplan 2 microscope with 10x 0.3 NA objective. The fluorescence intensity of polyubiquitin reporter animals was quantified using the free image quantification software Fiji. Immunostained polyubiquitin reporter animals were imaged with a motorized Zeiss AxioVert 200M inverted confocal microscope with LSM 510 point scanner. LSM software release 3.2 and 63x 1.4 NA plan-apochromat objective were used. *uchl5* transfected U-2 OS cells and ataxin3(Q28)-GFP or ataxin3(Q84)-GFP expressing constructs were imaged with Zeiss Axioplan 2 microscope with 20x 0.5 NA objective. Cells expressing UbG76V-GFP treated with siRNA were imaged with 10x 0.3 NA objective. Fluorescence intensities and aggregate counts were analyzed in a pipeline created in the Anduril analysis framework [561].

2. Biochemical methods used in I

2.1. Immunofluorescence in *C. elegans*

A mixed population of animals of varying age expressing the intestinal *PVha-6::UIM2-ZsProSensor* reporter was fixed and permeabilized, according to the protocol described by *Finney and Ruvkun*, 1990 [562]. Polyubiquitinated proteins were visualized with the FK1 antibody (Enzo Life Sciences) with an Alexa Fluor 594-conjugated secondary antibody (Invitrogen).

2.2. RNA interference (RNAi) in *C. elegans* by feeding

Unless otherwise stated, RNAi was performed according to the standard feeding protocol described by *Timmons et al*., 2001 [563]. The normal food source of the animals is replaced by bacteria containing a plasmid for the gene of interest, or only the empty control vector. Experiments utilizing RNAi clones from the J. Ahringer library used the HT115 *E. coli* strain carrying an empty pL4440 expression vector as control. The *rpn-2* RNAi (C23G10.4, J. Ahringer library) RNAi on *daf-2(e1370)* mutants carrying intestinal *UbG76V-Dendra2* was started at two days old (L3 larvae), two days before imaging. The *rpn-2* RNAi for RNAi sensitive *rrf-3(pk1426)* animals was started at three days old (L4 larvae), and animals were harvested two days later for proteasome *in-gel* activity assays. The *rpn-2* RNAi on animals expressing polyubiquitin reporter in the intestine was started at three days old (L4 larvae), and the animals were imaged after three days.

Bacteria carrying empty RNAi vector (pL4440) were used to dilute *ubh-4* RNAi bacteria for relevant experiments. RNAi against *ubh-4* and *lgg-1* (C08B11.7 and C32D5.9, J. Ahringer library) were started at one day old (L1 larvae) for imaging. *ubh-4* RNAi was initiated at the same stage also in proteasome *in-gel* activity assays, lifespan experiments and progeny counts. The RNAi constructs for *daf-16* (pAD43) and pAD12 (control) were generous gifts of Dr. Andrew Dillin. Induction of the plasmids were achieved by adding 10m isopropyl b-D-1-thiogalactopyranoside (IPTG) in the growth media. IPTG was added to a final concentration of 20 mM before seeding the bacteria on NGM agar plates (containing 100 μg/ml Amp + 12,5 μg /ml Tet + 0,4 mM IPTG). *L4440-daf-16a* (plasmid 31503) and *L4440-daf-16 df* (plasmid 31505) RNAi expression vectors [564] were purchased from Addgene. RNAi against *daf-16* was initiated at one day old (L1 larvae) in all experiments, and all animals were imaged at four days old (young adult stage).

2.3. Immunoprecipitation

Animals from three large agar plates were collected into M9 buffer into a final volume of 1.5 ml for UBH-4 immunoprecipitation. Dounce homogenizer (7 ml, tight pestle) was used for animal lysis in native-gel lysis buffer, using

approximately 150 pushes with the Dounce [565]. Following product instructions, His-tag immunoprecipitation was performed with 50 µl of Protino Ni-NTA agarose (Macherey-Nagel). Resulting eluates were analyzed by Western blot.

2.4. Native proteasome and deubiquitinase activity *in-gel* assays

Four days old (young adult) animals age-synchronized with hypochlorite treatment were collected in M9 buffer and frozen in -80 °C overnight. A Dounce homogenizer was used for lysing the collected samples in native-gel lysis buffer [565]. *In-gel* proteasome activity assay used suc-LLVY-AMC (Bachem) as the substrate. Colloidial Blue Staining Kit (Invitrogen) was used for Coomassie staining to assess sample loading. Deubiquitinating assay was performed in similar manner as proteasome *in-gel* activity assay, but the substrate was substituted with ub-AMC (Boston Biochem). Photoshop 9.0 (Adobe Systems) was used for adjusting signal levels and Fiji was used for quantification.

2.5. Western blotting

Young adult animals age-synchronized with hypochlorite treatment were collected in M9 buffer and frozen in -80 °C overnight. Samples were changed into Laemmli sample buffer [566] and lysed by alternate boiling and freezing, or by sonication in lysis buffer (50 mM Hepes (pH 7.4); 150 mM NaCl, 5 mM EDTA; 20 mM NEM and protease inhibitor tablet (Roche)), or with using native-gel sample lysis method. 7.5% and 10% polyacrylamide gel were used for sample separation. The gels were blotted onto nitrocellulose membrane with a semi-dry blotting system (BioRad Laboratories).

Antibodies used were: anti-α-tubulin antibody (Sigma), FK1 antibody for polyubiquitinated proteins (Enzo Life Sciences), proteasome 20S α1,2,3,5,6,7 antibody (Enzo Life Sciences), Anti-His antibody (Qiagen) and anti-HA antibody (Covance). Streptavidin-HRP conjugate (Dako) was used in blotting. Photoshop 9.0 (Adobe Systems) was used to adjust Western blot signal levels, which were then quantified by the free image analysis software Fiji.

2.6. Quantitative real-time PCR (qPCR)

Age-synchronized four days old (young adult) animals were collected and used for RNA extraction with the NucleoSpin RNA II kit (Macherey-Nagel) or GeneJet RNA Purification Kit (Fermentas). Maxima First Strand cDNA Synthesis kit for RT-qPCR was used for cDNA synthesis. Quantitative real-time PCR was performed with Maxima® SYBR Green/ROX qPCR Master Mix (2x) (Fermentas), using LightCycler 480 qPCR machine (Roche). **Table 5** lists the oligos used in qPCR analyses.

Table 5. qPCR oligos.

Gene (*C. elegans*)	Forward	Reverse
ubh-4	acttgttccaaaccgcaaac	tttccttgcggtacatttcc
act-1	tcggtatgggacagaaggac	catcccagttggtgacgata
cdc-42	ctgctggacaggaagattacg	ctcggacattctcgaatgaag
pmp-3	gttcccgtgttcatcactcat	acaccgtcgagaagctgtaga
sod-3	ggagttctcgccgtccg	gtcgaatgggagatctgggag
hsp-12.6	tggagttgtcaatgtcctcg	gacttcaatctcttttgggagg
mtl-1	agtgtgactgcaaaaacaagcaa	tccactgcattcacatttgtctc
fat-7	atagtgtggcgtaacgtggc	tagagagcaaatgagaagacg
aft-5	ccatcaatcttatcaacagcatcat	ctggtggaaccgaagtg
haf-7	gacgtggaaaagctgagagg	gcagggaaaatgtgaggaaa
gst-10	gtctaccacgttttggatgc	actttgtcggcctttctctt
gst-4	cccattttacaagtcgatgg	cttcctctgcagtttttcca
gcs-1	aatcgattcctttggagacc	atgtttgcctcgacaatgtt
rpn-6.1	gccgatttgattcgtgaact	caccagtgagagaagcacga
Gene (human)	**Forward**	**Reverse**
uchl5	gccagttcatgggttaattttt	atcgtgtcaagtcgggagtc
gapdh	acccagaagactgtggatgg	ttcagctcagggatgacctt

*Table modified from **Table S7** of **(I)**.*

3. Materials and methods used in publications II-IV

3.1. Patients

The patient cohorts (including the relevant ethical permit numbers) are described in more detail in their respective publications (**II-IV**).

*3.1.1. Patients in publication **II***

The patient cohort comprised 154 PDAC patients who underwent elective surgery between 2000-2011 at the Department of Surgery, Helsinki University Hospital, Finland. No other variants of exocrine pancreatic cancer were included in the study. Median age of patients was 64 (range 39-83), with a median follow-up of 2.0 (range 0.2-13.1) years. Patient records were used to gather clinical data, survival data was provided by the Finnish Population Registry, and cause of death by Statistics Finland [567-569].

*3.1.2. Patients in publication **III***

The patient cohort comprised of 840 CRC patients treated between 1983-2001 at the Department of Surgery, Helsinki University Hospital, Finland. Patient median age was 66, with a median follow-up of 5.1 years (range 0-25.8). Patient records were used to gather clinical data, follow-up data was provided by the Finnish Population Register Centre, and cause of death was provided by Statistics Finland. [570]

*3.1.3. Patients in publication **IV***

The patient cohort comprised of 650 gastric cancer patients treated between 1983-2009 at the Department of Surgery, Helsinki University Hospital, Finland. Patient median age was 66.9 (interquartile range 57.0-75.0), with a median follow-up time of 1.6 (interquartile range 0.6-4.7) years. Patient records were used to gather clinical data, while survival data was acquired from the Population Register Centre of Finland and cause of death was provided by Statistics Finland. [571,572]

3.2. Preparation of tumor tissue specimens

Tumor samples from the archives of the Department of Pathology, Helsinki University Hospital were formalin-fixed and paraffin-embedded. An experienced pathologist marked representative tumor areas on hematoxylin- and eosin-stained tumor slides (**II-IV**), and further re-confirmed PDAC histopathological diagnosis (**II**). A semiautomatic tissue microarray instrument (TMA) (Beecher Instruments, Silver Spring, MD) was used to acquire and mount either three 0.6 mm (**IV**) punches, and six (**II**), three (**III**) or four (**IV**) 1.0 mm punches from each donor block on the recipient TMA blocks.

3.3. Antibodies for immunohistochemistry

Rabbit anti-UCHL5 antibody (Sigma Aldrich HPA005908) was used for immunohistochemical stainings (**II-IV**). A small subset of samples was validated with a second anti-UCHL5 antibody (Santa Cruz sc-271002 (**II-IV**)). Additionally, a third anti-UCHL5 antibody (Sigma Aldrich SAB1400553) was used for similar validation (**III**).

3.4. Immunohistochemistry

TMA- and tissue blocks were freshly cut into 4 μm sections. Deparaffinization of the slides was performed with xylene, and rehydration achieved through a decreasing ethanol and distilled water series. For antigen retrieval, slides were treated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in Tris-HCl (pH 8.5) buffer for 20 min. at 98°C. Autostainer 480 (Lab Vision) by Dako REAL EnVision Detection system, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) was used for staining of the sections. Primary antibody was incubated for 1 hour at room temperature.

3.5. Sample scoring and imaging

UCHL5 expression was scored according to tumor-staining intensity as 0 (undetectable staining, negative), 1 (low staining), 2 (moderate staining), or 3

(strong staining) (**II-IV**). In PDAC, nuclear intensity was scored separately from cytoplasmic staining according to the proportion of positive nuclei present in the tumor tissue: 0%-10% positive nuclei scored as 0, 11%-40% as 1, 41%-75% as 2, and 76%-100% as 3 (**II**). Scoring was performed independently without knowledge of patient identity or disease outcome, first by one researcher, and then confirmed by an experienced pathologist. Any differences in sample scoring were discussed until a consensus was reached. Randomly chosen staining samples were chosen for representative images (**II-IV**).

3.6. Statistical analysis

Similar methods were used in all three clinical papers (**II-IV**) for statistical analysis, and in all three a large part of the analysis was performed by the same biostatistician. However, as in all three studied cancer types UCHL5 immunoexpression was handled slightly differently after scoring, and as all cancers record varying relevant clinicopathological traits, the statistical analysis performed on the material are here described separately.

II: UCHL5 expression was dichotomized for statistical purposes. Cytoplasmic UCHL5 immunoexpression was divided into low (scores 0-1) and high (scores 2-3) staining, and nuclear immunoexpression into either negative (< 10% nuclear positivity, score 0) or positive (> 10%, scores 1-3) staining. Median score of each patient was used in further analysis, because expression values in pancreatic tumor tissue often show great variation. Fisher's exact test and linear by linear association was used to evaluate association of UCHL5 expression and clinicopathological parameters. The Kaplan-Meier method was used for survival analysis, and groups were compared with the Breslow test. Uni- and multivariate survival analysis was analyzed with the Cox regression proportional hazard model adjusted for age, gender, stage, metastasized lymph-node ratio (LNR) ≥/<20% (cut-off ≥/<20%), and postoperative adjuvant therapy. A combination variable was generated for multivariate analyses because stage and LNR are internally correlated. Interaction terms were considered. The assumption of constant hazard ratios over time for the Cox model was tested. For each testable variable at a time, a time-dependent covariate was included separately. All variables fulfilled the assumption. A p value of less than 0.05 was considered significant, with all tests two-sided. Analysis was performed on SPSS version

24.0 (IBM SPSS Statistics, version 24.0 for Mac; SPSS, Inc., Chicago, IL, USA, an IBM Company).

III: The four scoring categories (0-3) of UCHL5 immunoexpression were kept separate for statistical analysis. Fisher's exact test and linear by linear association test (with Monte Carlo estimation of exact *p* values) was used to evaluate association of UCHL5 expression and clinicopathological parameters. Cumulative survival was evaluated by Kaplan-Meier survival analysis, and differences between groups were compared with the *log rank*-test. A *post-hoc* analysis with individually comparing UCHL5 expression levels 0-2 to the high expression level (3) one by one was used, if the overall survival comparison between expression levels of UCHL5 was significant. The Šidák correction was applied to multiple comparisons. The 5- and 10-year survival rates and mean survival times were calculated with confidence intervals (CI, 95%). For model uni- and multivariate analysis, Cox proportional hazard regression was performed on survival data. A time-dependent variable for each testable variable was added to test the Cox model assumption of constant hazard ratios over time. Hazard ratios of Dukes D class and differentiation status were analyzed in two time-periods with the time-dependent Cox model to fulfil the Cox model assumption. Interactions were considered, but no significant interaction emerged. A *p* value less than 0.05 was considered significant, and two-tailed tests were used. Statistical analysis was performed with IBM SPSS statistical package version 22 (IBM, New York, NY, USA) and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

IV: UCHL5 expression was dichotomized for statistical purposes into either negative (score 0) or positive (scores 1-3), and the maximum score was used in the statistical analysis. Fisher's exact test and linear by linear association test (with Monte Carlo estimation of exact *p* values) was used to evaluate association of UCHL5 expression and clinicopathological parameters. Cumulative survival was evaluated with Kaplan-Meier survival analysis, and differences between groups were compared with the *log rank*-test. Survival rates were calculated with exact 95% confidence intervals (CI). The Cox regression proportional hazard model was used for uni- and multivariable survival analysis, and multivariable model was adjusted for age, gender, stage and tumor size ($\geq$ 5 cm vs. <5 cm). The assumption of constant hazard ratios over time for the Cox model was tested. For each testable variable, a time-dependent covariate was included separately

one at a time. Except for one multivariable model, all variables fulfilled the assumption. Interaction terms were considered. A *p* value of less than 0.05 was considered significant, and two-tailed tests were used. Statistical analyses were performed with SPSS version 24.0 (IBM SPSS Statistics, version 24.0; SPSS, Inc., Chicago, IL, USA, an IBM Company, and with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

1. Proteasome-associated deubiquitinase UCHL5/UBH-4 is a modulator of proteasome activity in *C. elegans* and human cancer cells (I)

1.1. Insulin/IGF-1 signaling regulates proteasome activity in a tissue-specific manner through DAF-16/FOXO in *C. elegans*

In a previous work from our laboratory, a tissue-specific change in proteasome activity was observed in aging *C. elegans* [558]. The degradation of the photoconvertible UPS activity reporter (UbG76V-Dendra2, see Methods 1.2.) was diminished in the neuronal dorso-rectal ganglion, but not in the body-wall muscle cells of one week old animals. To explain this observation, we decided to study the potential role of Insulin/ Insulin-like growth factor 1 (IGF-1) signaling (IIS) in modulation of proteasome activity in *C. elegans*. IIS is a well-established aging-regulatory pathway, modulating lifespan and various stress responses in *C. elegans* and higher organisms [257,573]. **Figure 4** illustrates a simplified version of the IIS pathway, and names some of the key members in humans and *C. elegans*. The IIS cell-surface receptor in *C. elegans* is DAF-2. Long-lived *daf-2(e1370)* null-mutants exhibit reduced IIS, resulting in approximately two-fold expansion in lifespan and increased stress resistance [574-576]. IIS negatively regulates the transcription factors DAF-16/FOXO and SKN-1/Nrf [577-580]. Under reduced

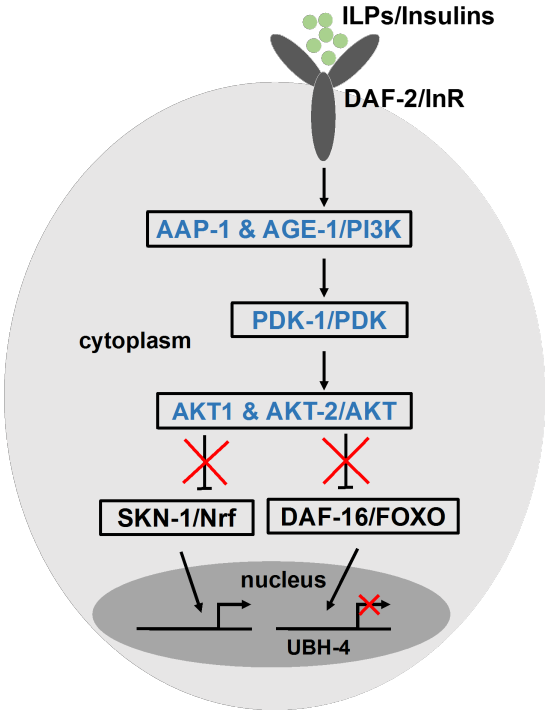

Figure 4. Insulin/IGF-1 signaling (IIS) in *C. elegans* and humans. Modified from *Sun et al., 2017.* [581]

IIS, DAF-16/FOXO becomes localized to the nucleus and constitutively active [258]. DAF-16 is also required for the lifespan extension seen in *daf-2(e1370)* mutants, and DAF-16 null mutants have a shortened lifespan [579, 581]. Although many studies have linked IIS to UPS regulation (*e.g.* [279,297]), it was unknown whether the proteasome itself is regulated by IIS.

Using our UPS-activity reporter, we observed an increase in its degradation in both the intestine (**I: Figure 1A** and **1B**) and body-wall muscle cells (**I: Figure 1D**) of long-lived *daf-2* mutant animals. In the intestine, this increase in proteasomal activity was found to be DAF-16 dependent, where the UPS-activity reporter degradation in short-lived *daf-16;daf-2* double mutants was comparable to wild type animals (**I: Figure 1A** and **1B**). Intriguingly, different *daf-16* mutants exhibited ambiguous results in body-wall muscle cells in degradation of the UPS-activity reporter. Altogether eight DAF-16 isoforms have been described in *C. elegans*, which are expressed differently in various tissues, and convey different functions [582]. The short-lived DAF-16 mutants used in (**I**) were *daf-16(m26)*, which lacks five isoforms, and the DAF-16 null-mutants *daf-16(mgDf47)* and *daf-16(mgDf50)*. Short-lived *daf-16(m26)* mutants displayed similar reporter degradation rate as the long-lived *daf-2* mutants (**I: Figure 1D**). In contrast, short-lived *daf-16(mgDf50)* null-mutants had a reporter degradation rate similar to that of wild type animals (**I: Figure 1D**). Further, the short-lived *daf-16(m26);daf-2(1370)* double mutants exhibited UPS activity that was not significantly different from either *daf-2(1370)*, or wild type animals, but falling somewhere in between the two (**I: Figure 1D**). Last, in the second short-lived *daf-16(mgDf47);daf-2(1370)* double mutant, UPS-activity reporter degradation was again comparable to degradation rate exhibited by *daf-2(1370)* mutants (**I: Figure 1D**). Taken together, it appears that in *C. elegans* body-wall muscle cells, IIS regulates UPS-activity through different DAF-16 isoforms than in the intestine, and in addition through other yet unidentified factor(s). This creates another interesting level into the UPS-activity regulation mediated by IIS.

As it is possible that the degradation of the UPS-activity reporter is affected by specific E3 ubiquitin ligases, we used our second *in vivo* reporter (*ZsProSensor::UIM2*, see Methods 1.2) that binds to endogenous polyubiquitinated proteins to complement the results. RNAi against *daf-16* increased reporter amount in the intestine of long-lived *daf-2* animals, but not in the wild type background (**I: Figure 2**). This confirmed the importance of DAF-

16 for activating proteasomal degradation in the intestine. Downregulation of *daf-16* with RNAi in the long-lived *daf-2* mutants is to some extent analogous to the inactive DAF-16 state present in the wild type background, which could cause accumulation of polyubiquitinated proteins reflected by the reporter. Further, a biochemical approach using *in-gel* proteasome activity assay (protocol according to [565]) also showed increased proteasome catalytic activity under reduced IIS in a DAF-16 dependent manner (**I: Figure 3A** and **3B**). In the long-lived *daf-2* mutants, there was an increase in the degradation of the 20S CP substrate (suc-LLVY-AMC), whereas the short-lived *daf-16* and *daf-16;daf-2* double mutants exhibited activity closer to wild type animals (**I: Figure 3A** and **3B**). However, this assay is not tissue-specific, as it uses native proteasome complexes isolated from whole animal-lysates.

Remarkably, though we observed increase in degradation in the long-lived *daf-2* mutants at multiple fronts, we could detect no difference in the amount of proteasome α-subunits (**I: Figure 3C** and **Figure 3D**). Western blot performed from whole animal lysates displayed no significant differences in proteasome amounts between long-lived *daf-2* mutants, short-lived *daf-16* mutants, nor short-lived *daf-16;daf-2* double mutants, when compared to wild type animals (**I: Figure 3C** and **Figure 3D**). In support of our observation, neither gene expression profiling or microarray proteome analysis with mass spectrometry has detected DAF-16-induced proteasome subunit expression in long-lived *daf-2* mutants [583-585]. Further, in a recent study from our laboratory, using formalin-fixed, paraffin-embedded *C. elegans* sections, no difference in proteasome tissue immunoexpression was seen in the long-lived *daf-2* mutants, compared to wild type animals [586]. This suggests that the DAF-16-mediated increase in UPS activity was not a result of direct changes in proteasome abundance.

1.2. The proteasome-associated deubiquitinase UBH-4 is a DAF-16 target gene

As there was no detectable difference in proteasome amounts in the long-lived *daf-2* mutants, we postulated that there could be differences in proteasome composition. Mass spectrometry (LC-MS/MS) analysis was performed by an outside source on proteasome complexes isolated in native gel, using samples from both long-lived *daf-2* mutants and wild type animals. The most notable difference between the samples was the presence of the proteasome-associated

DUB UBH-4 in wild type animals, absent from *daf-2* mutants (**I: Supplementary Table 3**). The association between 19S subunit RPN-13 and UBH-4 previously identified *in vitro* for *C. elegans* [587] was confirmed *in vivo* by immunoprecipitation (**I: Figure 4A**). UBH-4 DUB-activity in *C. elegans* was also corroborated using native *in-gel* assay, where knockdown of *ubh-4* with RNAi lead to decreased degradation of the DUB-specific ub-AMC substrate (**I: Figure 4B**). Using transcriptional reporter animals, we observed that wild type animals expressed UBH-4 in all tissues, and most notably in the intestine (**I: Figure 5A**, the first row of panels from the left). In contrast, the intestine of long-lived *daf-2* mutants was conspicuously lacking in UBH-4 expression (**I: Figure 5A**, the second row of panels from the left). Both short-lived *daf-16* mutants and *daf-16;daf-2* double mutants had a similar expression pattern with wild type animals (**I: Figure 5A**, the four panels on the right). One canonical DAF-16 binding site was found on the *ubh-4* promoter region. Mutation of this binding site re-established UBH-4 transcriptional reporter expression in the intestine of long-lived *daf-2* mutants (**I: Figure 5B**). Together, this supports a role for DAF-16 as a negative regulator of UBH-4 expression, especially in the intestine in *C. elegans*.

1.3. UBH-4 modulates proteasome activity and life history traits in *C. elegans*

Lower UBH-4 expression and higher proteasome activity were observed in the long-lived *daf-2* mutants most prominently in the intestine. The lifespan expansion exhibited by these animals is also significantly dependent on DAF-16 activity in the intestine [588]. Therefore, we postulated that UBH-4 functions as a proteasome inhibitor under normal IIS, probably in a tissue-specific manner. A similar inhibitory role has already been established for USP14/Ubp6 in delaying proteasomal degradation [218,220,589]. It is also known that the mammalian orthologue UCHL5 trims substrate ubiquitin chains, at times saving them from degradation [219,220,590]. When measured by qPCR, long-lived *daf-2* mutants exhibited approximately 20% lower *ubh-4* messenger RNA (mRNA) level than wild type animals (**I: Figure 5C**). Both short-lived *daf-16* and short-lived *daf-16;daf-2* double mutants had *ubh-4* mRNA level comparable with wild type animals, supporting our conclusion that DAF-16 negatively regulates UBH-4 expression. Hoping to artificially mimic the reduced UBH-4 levels present in *daf-2* mutants, we mixed *ubh-4* RNAi with control bacteria containing only the empty

pL4440 vector. As a result, *ubh-4* RNAi diluted to 1% concentration with the control bacteria induced a knock down of UBH-4 levels to 80% in wild type animals (**I: Figure 5D**).

We performed a series of experiments with both undiluted and diluted (1%) *ubh-4* RNAi. In the intestine, we observed higher UPS-activity reporter degradation following both undiluted and diluted *ubh-4* RNAi (**I: Figure 6A**). Similarly, the intestinal polyubiquitin reporter displayed decreased accumulation of polyubiquitinated proteins after both undiluted and diluted *ubh-4* RNAi (**I: Figure 6C**). The *in-gel* activity assay performed from whole animal lysates showed an increase in proteasome catalytic activity after undiluted *ubh-4* RNAi (**I: Figure 6D**). In contrast, undiluted *ubh-4* RNAi decreased degradation of the UPS-activity reporter compared to the wild type in body-wall muscle cells, and reporter degradation after diluted *ubh-4* RNAi was comparable with the wild type in these animals (**I: Figure 6B**). Further, undiluted *ubh-4* RNAi also increased accumulation of polyubiquitinated substrates by Western blot in whole animal lysates (**I: Figure 4C** and **Figure 4D**). Taken together, UBH-4 appears to tissue-specifically inhibit the proteasome. Following *ubh-4* RNAi, polyubiquitinated substrates are accumulated in some tissue(s), which exhibits as accumulation of proteasome substrates on organismal level. It is possible that UHB-4 levels fluctuate along a dynamic range. Too high UBH-4 level inhibits the proteasome in disproportionate amounts, whereas too low UBH-4 level is in other ways problematic. This could be caused in part by the role of UBH-4 in ubiquitin-chain trimming [219], which could assist in keeping undegradable substrates from becoming stalled on the proteasome. Conversely, UBH-4 could also save lightly ubiquitinated substrates from being destroyed, while promoting the degradation of more heavily ubiquitinated proteins. The optimal range of UBH-4 expression is almost certainly tissue-specific, and probably alters in response to prevailing cellular conditions, *e.g.* reduced IIS. Additionally, the decreased degradation of the UPS activity reporter observed in body-wall muscle cells after undiluted *ubh-4* RNAi could also be caused by a decline in overall physiology, as the animals appeared outwardly unwell during imaging.

We were also interested in how UBH-4 might affect different *C. elegans* life history traits, such as lifespan and progeny amounts. A previous genome-wide RNAi screen had shown that *ubh-4* RNAi slightly increases lifespan [591]. However, undiluted *ubh-4* RNAi resulted in consistent lifespan reduction of the

RNAi sensitive *rrf-3(pk1426)* mutant used as the wild type in our lifespan experiments (**I: Figure 6F** and **Supplementary Table 4**). The different result could be dependent on either RNAi efficacy, or the different temperature used in the experiments, which in the case of *Hamilton et al*. constitutes a mild heat stress for the animals (25 °C compared to 20 °C used in our experiments) [591]. In contrast, diluted *ubh-4* RNAi produced a slight but consistent increase in lifespan of the *rrf-3* mutants (**I: Figure 6F** and **Supplementary Table 4**). A similar effect was also evident in progeny amounts: undiluted *ubh-4* RNAi decreased offspring amounts, whereas diluted *ubh-4* RNAi had no effect (**I: Supplementary Table 5**).

In the long-lived *daf-2* mutants and the short-lived *daf-16* mutants, both undiluted and diluted *ubh-4* RNAi had no effect on animal lifespan (**I: Figure 6F** and **Supplementary Table 4**). Further, intestinal overexpression of UBH-4 slightly decreased *daf-2* mutant lifespan (**I: Figure 6H** and **Supplementary Table 4**). Taken together, the results indicate that for *daf-2* mutants, the decrease in UBH-4 expression is important for at least a part of the lifespan extension normally observed in these animals. On the other hand, the normally observed levels of UBH-4 in the *daf-2* mutants may already optimal, so further knockdown of *ubh-4* by RNAi has no cumulative positive effect on their lifespan. In addition, activation of other DAF-16 target gene(s) are likely needed for the diluted *ubh-4* RNAi-mediated lifespan extension, as there was no change in lifespan in the short-lived *daf-16* mutants (**I: Figure 6F** and **Supplementary Table 4**). It appears that correct level of UBH-4 expression is important for achieving the observed slight lifespan extension in the RNAi sensitive *rrf-3* animals. This tissue-specificity and balance in the levels of IIS signaling needed for increased lifespan and proteostasis has been described elsewhere [260]. The apparent existence of tissue-specific optimal level of UBH-4 beneficial for the organism implied by our results support this finding.

1.4. Knockdown of *uchl5* promotes degradation in mammalian cell lines

Our promising results with UBH-4 prompted interest in its mammalian orthologue, UCHL5. We inhibited PI(3)K, an important component of the IIS pathway, in human osteosarcoma cells (U-2 OS), thus causing an approximately 30% knockdown of *uchl5* mRNA levels (**I: Figure 7A**). Therefore, IIS appears

to regulate human UCHL5 in a similar way that UBH-4 is regulated in *C. elegans*. The degradation of UPS-activity reporter UbG76V-GFP was also increased following *uchl5* siRNA-induced knockdown, demonstrating that UCHL5 regulates proteasome activity also in U-2 OS cells (**I: Figure 7B** and **Supplementary Figure 7A**). Finally, we observed increased degradation of ataxin3(Q28) and the proteotoxic ataxin3(Q84) following *uchl5* siRNA (**I: Figure 7C** and **Supplementary Figure 7**). UCHL5 could therefore have clinical relevance as a therapeutic target in protein aggregate-diseases, as a decrease in UCHL5 levels was shown to enhance UPS activity and degradation of proteotoxic proteins.

2. UCHL5 is a prognostic marker in three gastrointestinal cancers (II-IV)

Following our discovery that *uchl5* downregulation increases degradation of proteotoxic proteins in human cancer cells (**I: Figure 7C** and **Supplementary Figure 7**), we became curious of the role of this DUB in different gastrointestinal cancers. Our interest was further increased by the fact that the effects of UBH-4 were particularly striking in the *C. elegans* intestine (**I: Figure 6A** and **6C**). UCHL5 levels are also reported to be elevated in *e.g.* breast, vulva and parathyroid cancers, and UCHL5 exhibited increased activity in cervical cancer [311]. For this thesis, UCHL5 immunoexpression was studied from patient tumor samples in three different gastrointestinal cancers: pancreatic ductal adenocarcinoma (PDAC, **II**), colorectal cancer (CRC, **III**) and gastric cancer (GC, **IV**). UCHL5 tumor expression, in association with clinicopathological variables and patient survival, were studied from all three cancer types, with promising results and with possible clinical potential.

2.1. UCHL5 tumor expression pattern varies depending on tissue of origin

UCHL5 tumor expression pattern, as measured with immunohistochemistry, was uniform and widespread throughout the tumor tissue cytoplasm in all three cancers, when present (**II-IV: Figure 1**). Interestingly, UCHL5 tumor expression pattern and levels showed a degree of tissue-specific variation in the studied cancers. Human Protein Atlas reports that UCHL5 tumor expression levels are predominantly moderate to low in all three studied cancer types [592].

Accordingly, in CRC and GC, moderate to low expression values comprised 79% and 69% of samples, respectively (**III** and **IV**). Further, in CRC and GC, only cytoplasmic staining was evaluated. In these two cancers, nuclear staining appeared to be either low, or masked by the ubiquitous cytoplasmic staining. This made it difficult to reliably assess if and to what degree nuclear staining was present in a majority of the samples, and therefore only cytoplasmic staining was scored.

In stark contrast, the majority (94.7%) of samples in PDAC were UCHL5 negative in the cytoplasm, or exhibited only low UCHL5 tumor staining (**II: Figure 1A** and **1B**). Conversely, the amount of UCHL5 positive nuclei showed high variation in PDAC tumor samples, or were possible to more accurately assess in the absence of potentially obscuring cytoplasmic staining. Positive (11%-100% positive nuclei) nuclear UCHL5 staining was scored in 51.7% of the samples, although only 5.9% exhibited high (76%-100% positive nuclei) UCHL5 expression (**II: Table 1**). Meanwhile, in the tumor-adjacent normal-appearing tissue, UCHL5 expression was predominantly low or negative in all three studied cancers (data not shown). In support of these findings, we observed tissue-specific variation also in *C. elegans* UBH-4 expression (**I: Figure 5A** and **5B**). Therefore, the possibly context-dependent and dynamic range of UCHL5 levels might reflect tissue-specific differences in UCHL5-mediated effects also in mammalian tissues.

In both GC and PDAC, UCHL5 tumor expression was dichotomized for further statistical analysis after scoring. In GC (**IV**), comparisons were made between UCHL5 tumor immunopositivity and negativity (0 vs. 3). In PDAC (**II**), nuclear UCHL5 staining was divided into positivity and negativity (0 vs. 3), whereas cytoplasmic staining was divided into low (scores 0-1) and high (scores 2-3). In contrast, in CRC (**III**) UCHL5 staining intensities associated with survival in an unconventional order, so that both high and negative UCHL5 tumor expression linked to increased survival. For this reason, the four scoring categories were kept separate for further analysis.

2.2. UCHL5 tumor expression correlates with cancer-specific survival in colorectal cancer, gastric cancer and pancreatic ductal adenocarcinoma

Significant cancer-specific survival (CSS) differences correlating with UCHL5 tumor expression were found in the whole patient cohort only in PDAC, where both high cytoplasmic ($p = 0.034$) and positive nuclear ($p = 0.005$) expression associated with better prognosis (**II: Figure 2A** and **2B**). However, though high cytoplasmic staining correlated with increased survival in PDAC, patients exhibiting this level of UCHL5 expression constituted only 8 patients (5.3% of the patient cohort). Corroboration of this result in other sample sets is required for making informed deductions about the role of cytoplasmic UCHL5 expression in PDAC. Additional subgroup analysis in PDAC patients revealed that distinct survival benefits associated with positive UCHL5 nuclear expression, *e.g.* in patients with regional disease (stages IIB-III, $p = 0.007$) and 65 years or older ($p = 0.001$) (**II: Figure 3A** and **3B**).

Importantly, subgroup analysis revealed that UCHL5 tumor expression associated significant differences in CRC and GC patient survival. In lymph node-positive (Dukes C/stage III) rectal cancer, patients with both strong and negative UCHL5 tumor expression exhibited increased survival ($p = 0.012$) (**III: Figure 3**). In gastric cancer, positive UCHL5 tumor expression-linked increased survival were seen in patients with small tumors (<5 cm, $p = 0.025$), stage I-II of the disease ($p = 0.001$) and those 66 years or older ($p = 0.037$) (**IV: Figure 2**). High UCHL5 expression has previously been linked to decreased survival and cancer recurrence in hepatocellular carcinoma, esophageal squamous cell carcinoma and epithelial ovarian cancer [303,309,310]. This is in direct contrast with our results, where high or positive UCHL5 expression was found to associate with better prognosis in PDAC, and subgroups of CRC and GC patients. This could be due in part to differences in the methods used (Western blot; [303,310]) and analysis methods. Most importantly, as demonstrated earlier, UCHL5 displays a high-degree of tissue-specificity regarding its expression, which could also explain the observed difference.

2.2.1. UCHL5 tumor expression association with cancer stage

Further, increased CSS associated with positive UCHL5 tumor expression appeared to correlate with stage in all three studied cancer types. In PDAC and

CRC, patients with more serious stage of the disease (Dukes C/stage III in CRC; stage IIB-III in PDAC) and high or positive UCHL5 expression appeared to have better survival, whereas in gastric cancer patients with lower stage of the disease and positive UCHL5 expression had better prognosis. The 5-year CSS for Dukes C/stage III CRC patients with high UCHL5 expression was 100% and for patients with negative expression 84.4% (95 CI 49.3%-96.0%) (**III: Table 2**). In comparison, for patients with either low or moderate UCHL5 expression, the 5-year CSS was only 42.5% (95% CI 27.3-56.8) and 47.6% (95% CI 29.0-64.0), respectively (**III: Table 2**). Further, none of the patients with high UCHL5 expression had died from rectal cancer even 10 years after surgery **(III: Table 1)**. However, it should be noted that in this subgroup of Dukes C/stage III rectal cancer, only 7 patients originally exhibited high UCHL5 tumor expression (**III: Table 2**).

In regional (stages IIB-III) PDAC, 2-year CSS in patients with positive nuclear UCHL5 tumor expression was 56.2% (95% CI 41.4-68.6)), compared to 34.0% (95% CI 21.7-46.7) in patients with nuclear UCHL5 negativity (**II: Table 2**). The difference in 5-year CSS for these patients was additionally almost two-fold, with 19.9% (95% CI 10.2-31.9) for UCHL5 positive and 10.4 (95% CI 3.8-20.8) for UCHL5 negative patients, respectively (**II: Table 2**). In contrast, GC-patients with lower (stages I-II) stage of the disease and with positive cytoplasmic UCHL5 tumor expression had 5-year CSS of 77.2% (95% CI 69.2-83.4), compared to 59.0% (95% CI 41.3-73.0) in patients with no cytoplasmic UCHL5 tumor expression (**IV: Table 2**). This may reflect the tissue-specificity that UCHL5 displays in its expression (**II-IV: Figure 1**), and in its varied role in modulating normal cell functions, including proteasome activity (**I: Figure 7B and 7C**, **Supplementary Figure 7**). The observation that GC patients with small (<5 cm) tumor size and high UCHL5 expression have better prognosis might be linked with the increased survival seen in patients with positive UCHL5 expression and lower (stage I-II) stage of the disease (**IV: Figure 2B** and **2C**). There is doubtless a high degree of overlap between these two gastric cancer patient subgroups, as patients with lower disease stage also tend to have predominantly small tumors. Further, it has been established that UCHL5 and Rpn13 together are necessary for cell-cycle progression [238]. It is possible that low UCHL5 tumor expression inhibits proliferation in tumor cells, resulting in small overall tumor size.

2.2.2. UCHL5 tumor expression association with patient age

Age seemed to be another important factor in the UCHL5 tumor expression-associated survival benefit in both PDAC and GC. Older patients with positive UCHL5 expression had better prognosis in comparison to UCHL5 negative patients in these two cancer types (**II: Figure 3B** and **III: Figure 2B**). Many studies have shown reduced proteasome activity in elderly patients, and a decline in proteostasis is one of the hallmarks of aging [255]. However, there is still a lack of consensus if this concerns all tissues, and both Ub-dependent and Ub-independent proteasomal degradation [285]. Yet, assuming reduced proteasome-associated proteolytic capability in older patients, the increased UCHL5 levels may inhibit the proteasome in increasing amounts, thereby promoting apoptosis of cancer cells.

3. The potential roles of UCHL5 in association with its binding partners and a summary of the results

The key points of the results are summarized in **Figure 5**. Both UCHL5 and UBH-4 have a significant role in proteasome modulation, and a clear effect on certain lifehistory traits, *e.g.* patient survival in cancer, and lifespan in *C. elegans*. Across the three studied cancer types, a clear survival benefit was observed in patients with positive or high UCHL5 expression. However, the effect of UCHL5 might not be linked only, or possibly at all, with its association with the proteasome. As the 19S Rpn13 subunit is required for UCHL5 binding, all free 20S CPs lack UCHL5. Some studies suggest a high degree of free 20S in cells, particularly in response to stress [145]. In addition, not all conventional proteasomes contain Rpn13 [233], and it is possible Rpn13 is in only one of the 19S of a double-capped (RP-CP-RP) proteasome [593]. One study showed that approximately 60% of neuronal proteasomes in rats contained Rpn13 [594]. Human Rpn13 was also present in sub-stoichiometric amounts in purified proteasomes in two other studies [595,596]. Further, levels of Rpn13 and UCHL5 are decreased in response to nutrient deprivation [597], which is often a characteristic of tumor cells [338]. Increased inflammation of the tumor site and the surrounding stroma is another hallmark of cancer [338], inducing the expression of the immunoproteasome, which prefers to associate with PA28αβ activator instead of 19S [67,81]. Importantly, PA28 does not provide a binding

site for UCHL5. However, this does not exclude the possibility of hybrid proteasomes, capped at one end with a 19S accessible for UCHL5. In addition, Rpn13 is found overexpressed in colorectal cancer [598], gastric cancer [599], ovarian cancers and acute leukemia [600], potentially providing more available binding sites for UCHL5.

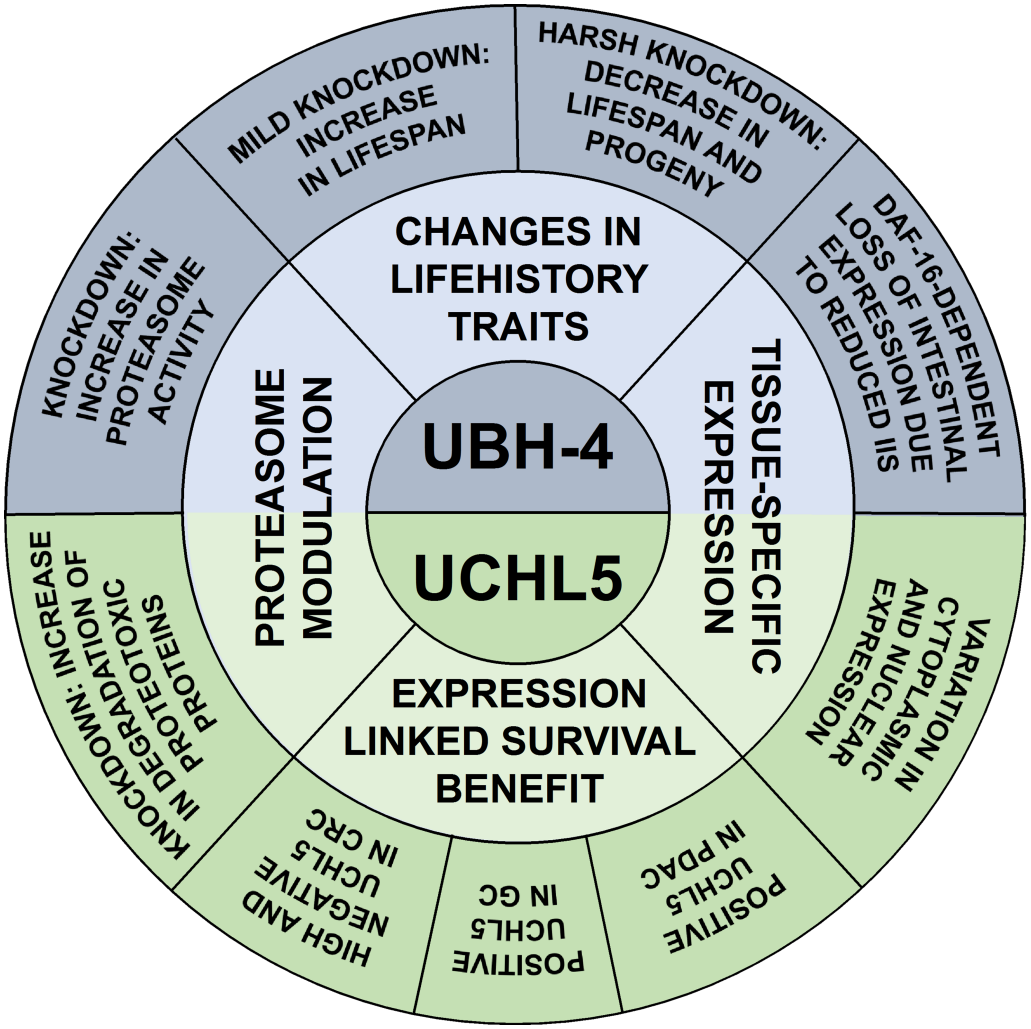

Figure 5. Summary of the results. The top hemisphere relates to UBH-4 and the lower one to UCHL5.

If the UCHL5-associated survival benefit is not linked with the proteasome, then it is possible that it stems from the association of UCHL5 with its other binding partners. UCHL5 is also a subunit of the chromatin remodeling complex INO80 [601,602], which belongs to the Sucrose Non-Fermenting 2 (SNF2) ATPase family [603]. Although the role of UCHL5 in the context of the INO80 complex is not well known, the complex has genome-wide significance in controlling chromatin structure and activity [604,605]. INO80 is involved in nucleosome turnover [606] and sliding [607], recruiting UCHL5 to the site [608]. HeLa INO80 complexes exhibit a high degree of UCHL5 association, and it has been suggested that UCHL5-free proteasomes are recruited to INO80 for target protein degradation on the chromatin through UCHL5 [235]. In PDAC, positive nuclear UCHL5 expression was an important factor in patient prognosis. However, the proteasome is found in both the nucleus and the cytoplasm. Therefore, UCHL5 expression observed in the nucleus is not enough to determine whether UCHL5 associated with INO80 or the proteasome in this instance. In the light of strong cytoplasmic UCHL5 expression present in CRC and GC, it seems unlikely that the UCHL5-associated survival benefit is linked solely to INO80. Finally, it should be noted that UCHL5 may function only as a surrogate marker, its expression following that of some yet unidentified factor(s), which in reality convey the survival benefit observed in our research. However, because UCHL5 displayed tissue-specific prognostic relevance in several cancer types, and because UCHL5 inhibition increases degradation of proteotoxic proteins in cancer cells, this also seems improbable.

CONCLUSIONS AND FUTURE PROSPECTS

In *C. elegans*, reduced IIS was associated with increased UPS activity in both the intestine and body-wall muscle cells. The DUB UBH-4 was demonstrated to modulate proteasome activity in tissue-specific manner with several complementing *in vivo* and biochemical assays. Further, the IIS downstream effector DAF-16/FOXO transcription factor was found to negatively regulate UBH-4 expression in the *C. elegans* intestine. Knockdown of mammalian *uchl5* also increased proteasome activity in cancer cells, prompting degradation of proteotoxic proteins. Taken together, it appears that at least a part of the increase in proteasome activity present under conditions of reduced IIS is mediated through the released inhibition of UBH-4 on the proteasome. However, other factor(s) are involved in mediating the effect of IIS on UPS activity, based on the tissue-specific responses caused by reduced IIS in *C. elegans*. As seen in the variable degradation of our UPS activity reporter in *daf-16* mutants in body-wall muscle cells, different DAF-16 isoforms mediate specific responses, and further IIS effector(s) remain to be identified. Other aging-signaling pathways may also regulate the UPS, and it would be interesting to study their potential role in modulating the proteasome. However, whatever details will be in uncovered in the future, the role of IIS in regulating proteostasis through UPS activity is important.

In a set of clinical studies using patient tumor samples, the human orthologue of UBH-4, UCHL5, was shown to have relevant prognostic value in three gastrointestinal cancers (rectal cancer, gastric cancer and pancreatic ductal adenocarcinoma). Significant survival differences were present in several patient subgroups; most prominently in the increased survival exhibited by lymph-node-positive (Dukes C/stage III) rectal cancer patients with high UCHL5 expression. Based on our results, UCHL5 is a promising prognostic marker for these cancer types, but more study is required to determine its clinical relevance, including possible potential as a therapeutic target. Several inhibitors are in development for targeting of DUBs in therapy, including proteasome-associated DUBs like UCHL5, with high hopes for less severe side-effects and other related adverse issues than are associated with conventional proteasome inhibitors. The validation of the prognostic potential of UCHL5 in gastrointestinal cancers is vital, especially if it is to be developed for use in clinical praxis. Corroborating our results in additional and especially international patient cohorts would be an

obvious next step to continue this project. Extension of studies to other cancer types could also provide interesting results, and further our understanding of the tissue-specific functions of UCHL5. At the same time, research into the molecular mechanisms causing the increased survival associated with UCHL5 expression are equally essential; for example, studying different processes associated with neoplastic formation and cancer progress in patent-derived tumor tissue organoid models, as mediated by UCHL5.

In addition, the significance of UCHL5 association with its other binding partners, including the INO80 chromatin remodeling complex, the TGF-β inhibitory SMAD7-SMURF2 complex, and possibly other yet unidentified factors remain unclear, also in the context of the UCHL5 mediated survival benefit. It should also be noted that several different UCHL5 isoforms are expressed by mammalian cells, and little is known about the prospective differences in their tissue-specific expression or function. Further studies are required to fully understand UCHL5 association and functionality in these different settings, and perhaps even cellular compartments. In conclusion, the specifics of the modulation of proteasome function, both in aging tissues and during different disease conditions, deserve more scientific attention. The results presented in this thesis may offer potential prognostic value for clinical use and help shape future research in proteostasis maintenance.

ACKNOWLEDGEMENTS

This work was carried out in the Translational Cancer Biology Research Program, Biomedicum Helsinki, at the University of Helsinki. It was funded by the Doctoral Programme in Biomedicine (DPBM), and by grants from the K. Albin Johansson Foundation, Syöpäsäätiö and Victoriastiftelsen.

I thank my supervisor, Docent Carina Holmberg-Still, for offering me this great opportunity to participate in an intriguing research project, which over time resolved itself into a PhD. Above all, I am grateful for her patient guidance over the past decade, and for making this thesis possible in the first place.

My extensive thanks go to my capable co-authors; this work would not have seen the light of day without them. Alli, Harri, Jaana, Kapo, Ville: thank you for your invaluable contributions. I thank Professor Caj Haglund for the existence of our fascinating collaboration projects, and also for agreeing to the role of kustos. Päivi Peltokangas and Olli-Matti Sirviö from Haglund group provided first-rate technical assistance. I thank also Sampsa Hautaniemi for our collaboration. I owe inmeasurable gratitute to Professor Hilde Nilsen for being my opponent; the defense will become have been educational//terrifying/fun. To Professors Nico Dantuma and Markus Mäkinen, I am grateful for their time and effort in reading the thesis, which was made all the better for their comments. I also thank Ville Hietakangas and Dan Lindholm for their support and help over the years as members of my follow-up committee.

To all current and former members of the Holmberg Lab: I collectively thank you for fun times and your endless help. Anni-Helena (AH), without you most of my projects would never have left the ground. Elisa and Sweta, thank you both for everything and good luck with your PhDs (not that you need it)! Geert, your assistance was essential at the beginning of my sciencing career, and I am eternally grateful. Johanna, my sincere thanks for your continuing help and tenacity, and in reading this thesis in progress (twice). Olli, you taught me much, and it could be argued this thesis is *your* fault; thank you for that. I'm also grateful to my worms, without whom much of this thesis would not exist. I am humbled by their sacrifice.

To my academic pals of the Biomedicum fame: Anita, Elsa, Jussi, Maija H., Maija S., Sussu, Tini: you have been there in the good and the bad; a thousand thanks to each. Elsa, your insight into EndNote was crucial. Jussi, your microscopy genius was instrumental for this thesis. Your GM genius is another story entirely; thank you for the nightmares. Maija H., we shared a lot of the same academic issues, and I thank you for all the emotional support. Maija S., you helped to keep me focused on the goal. Some commiserating *may* have taken place. Tini, you are the most optimistic person I've ever met, which of course is (sometimes) very annoying. How *dare* you not let me give up?!? *Thank you*.

In addition, there are many more people, whose ungrateful job it has been to conserve my sanity. Thank you all for your inhuman perseverance. Varja and Saara, thanks for the past 20 odd years; it has been fun! Tekla – the *bitch* to my *jerk*, *thank you*. Let's keep writing absurd stories for decades to come. Riikka – you literally gave me a roof over my head; my hearfelt thanks. Let's keep knitting! And please, mediate my appreciation (and some chicken) to Sam's indefatigable therapy dog, Huima. Jori – no one can create aberrant terror by a simple "Everything's ok" like you; that's a compliment, I think. Eevi – so *much* complaining entured by you from my part. Sorry & thank you. Special thanks also to Niina – I have enjoyed the repeated possibility of escapism to the country; please extend my well-wishes to worlds bestest Veikko and Janu.

Most of all, my thesis is dedicated to my family. My parents, besides providing my biological material, have for some inexplicable reason always believed in me. I never really even considered not pursuing an academic degree; that's all on *them*. My brothers, Jussi and Lassi, are aggravating troglodytes, but they will help you when you need – after first grumbling about it, of course. So, thanks! Samwise (or, depending on the day, Sammael the Hellhound) creates unending joy in my life (also gas, and rumpled bed clothes infused with sand). Live long and prosper, small dog! I would like to emphasize Tossu (the cat-shaped daemon) didn't do *anything*, except possibly lengthen the whole project a bunch, usually by doing something gross around 4 AM (she's vomiting as I write this).

To all of you, I owe my honest gratitude and heartfelt thanks, which most likely manifests in woolen socks somewhere down the line, just you wait… So long, and thanks for all the fish!

APPENDIXES

APPENDIX 1A. TNM staging of colorectal cancer after American Joint Committee on Cancer (AJCC), 7th edition.

Stage	T	N	M	Dukes'	ACPS
0	Tis	N0	M0	**-**	**-**
I	T1	N0	M0	**A**	**A**
	T2	N0	M0		
IIA	T3	N0	M0	**B**	**B**
IIB	T4a	N0	M0		
IIC	T4b	N0	M0		
IIIA	T1-T2	N1/N1c	M0	**C**	**C**
	T1	N2a	M0		
IIIB	T3-T4a	N1/N1c	M0		
	T2-T3	N2a	M0		
	T1-T2	N2b	M0		
IIIC	T4a	N2a	M0		
	T3-T4a	N2b	M0		
	T4b	N1-N2	M0		
IVA	Any T	Any N	M1a	**-**	**D**
IVB	Any T	Any N	M1b	**-**	

ACPS is the modified Dukes' staging (Australian Clinio-Pathological Staging, 1982) [409]. Modified from: http://emedicine.medscape.com/article/2006674-overview

APPENDIX 1B. T, N and M definitions in colorectal cancer.

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the pericolorectal tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum
T4b	Tumor directly invades or is adherent to other organs or structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to 1 organ or site (eg, liver, lung, ovary, nonregional node)
M1b	Metastases in more than 1 organ/site or the peritoneum

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APPENDIX 2A. TNM staging of gastric cancer after American Joint Committee on Cancer (AJCC), 7th edition.

Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
	T1	N1	M0
IIA	T3	N0	M0
	T2	N1	M0
	T1	N2	M0
IIB	T4a	N0	M0
	T3	N1	M0
	T2	N2	M0
	T1	N3	M0
IIIA	T4a	N1	M0
	T3	N2	M0
	T2	N3	M0
IIIB	T4b	N0	M0
	T4b	N1	M0
	T4a	N2	M0
	T3	N3	M0
IIIC	T4b	N2	M0
	T4b	N3	M0
	T4a	N3	M0
IV	Any T	Any N	M1

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APPENDIX 2B. T, N and M definitions in gastric cancer.

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades lamina propria or muscularis mucosae
T1b	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor penetrates subserosal connective tissue without invasion of visceral peritoneum or adjacent structures
T4	Tumor invades serosa (visceral peritoneum) or adjacent structures
T4a	Tumor invades serosa (visceral peritoneum)
T4b	Tumor invades adjacent structures
Regional lymph nodes (N)	
NX	Regional lymph node(s) cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-2 regional lymph nodes
N2	Metastasis in 3-6 regional lymph nodes
N3	Metastasis in seven or more regional lymph nodes
N3a	Metastasis in 7-15 regional lymph nodes
N3b	Metastasis in 16 or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

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APPENDIX 3A. TNM staging of pancreatic ductal adenocarcinoma after American Joint Committee on Cancer (AJCC), 7th edition.

Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

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APPENDIX 3B. TNM definitions in pancreatic ductal adenocarcinoma.

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor limited to the pancreas, ≤2 cm in greatest dimension
T2	Tumor limited to the pancreas, >2 cm in greatest dimension
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery
T4	Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor)
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

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